

University of Veterinary Medicine, Budapest
Postgraduate School of Veterinary Science
Budapest, Hungary

**Development of advanced antimicrobial combinations for
the treatment of canine otitis externa**

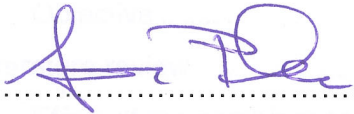
PhD Dissertation

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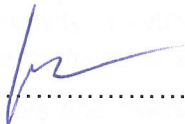
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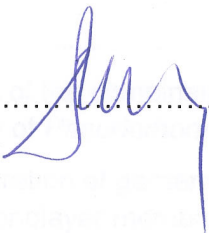


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List of abbreviations

AMR	Antimicrobial resistance
AMEG	Antibiotic Advice Ad Hoc Expert Group
CIA	Critically Important Antibiotics
HIA	Highly Important Antibiotics
DMSO	Dimethyl sulfoxide
FIC	Fractional Inhibitory Concentration
HIA	Highly Important Antibiotics
IPEC-J2	Porcine Intestinal Epithelial Cell J2
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MPC	Mutant Prevention Concentration
RONFA	Reduction of Need for Antimicrobials in Food-producing Animals
TEER	Transepithelial Electrical Resistance
VMP	Veterinary Medicinal Product

1. Summary

Given the high costs and necessary workload the development of new active substances for VMPs has been reduced. Furthermore, the use of antibiotics, which are considered CIA or HIA in human health are getting to be restricted or banned in the veterinary sector. It is assumed that appropriately selected antibiotic combinations that are synergistic may help to reduce the frequency of resistance and multiresistance. In case of *Escherichia coli* it has already been confirmed that use of certain antibiotics could promote sensitivity and hypersensitivity to other antibiotics even in case when hyper-resistance had already been observed before (Lázár *et al.*, 2013). It is also important to precisely determine the common effect of the fix combinations on the applied active ingredients and on the target animals for high safety aspect.

In this study I examined therefore some important correlations in antimicrobial combinations to suport the treatment of canine otitis externa and give information for the medicinal product development.

Our findings are summarized as follows:

Synergy has been found between marbofloxacin and gentamicin in 48.5% of the *P. aeruginosa* strains investigated, with a mean FIC index of 0.546 indicating only partial synergy for all of the strains. The threshold of full synergy is at 0.5 FIC, and no antagonistic effect was observed in none of the strains, therefore, according to our results, the justified and targeted usage of the marbofloxacin–gentamicin combination in infections caused by *P. aeruginosa* in the veterinary field may yield beneficial results, especially in topical products where the toxic effects of antibiotic might be negated if the tympanic membrane is intact.

Investigating the penetration, gentamicin showed poor paracellular permeation across the IPEC-J2 monolayer. Addition of 1% DMSO did not disrupt paracellular integrity and it did not alter the transport of gentamicin. Our results indicate that inclusion of DMSO in this low concentration as part of drug formulation does not promote oral absorption of gentamicin and it does not enhance the incidence of ototoxic effects caused by gentamicin in ear drop application. Gentamicin can also be used for detecting epithelial barrier dysfunction during LPS-induced bacterial infection or acute oxidative stress when chromatographic separation and quantitative fluorometric determination of

gentamicin collected from basolateral compartment of IPEC-J2 cell monolayer is performed.

In our 8-day passage investigation the minimum inhibitory concentrations (MICs) of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination in sixteen *P. aeruginosa* strains during the passages. Serial passage in subinhibitory concentrations showed a significant increase in the MICs of marbofloxacin after one day. Serial passage in subinhibitory concentrations showed an increase in the MICs of marbofloxacin–gentamicin (1:1) for all strains of *P. aeruginosa* but the MIC increase was much lower than that found for the treatment with marbofloxacin alone. No strain reached an MIC higher than 32 µg/ml.

We performed the 8-day passage examination with *S. pseudintermedius* bacteria too. Average multiplication of the MICs on day 8 passaged in marbofloxacin alone and the marbofloxacin–gentamicin (1:1) combination were 11.67 compared to 3.00 respectively. In addition it has to be stated that the original (1. day) results of the combination were usually lower, thus the multiplication factor indicates a much lower absolute increase. All of our results showed highly significant differences ($p < 0.0001$). Considering these results, it can be pronounced that the marbofloxacin–gentamicin (1:1) combination can significantly hinder the development of fluoroquinolone resistance in *S. pseudintermedius* bacteria isolated from canine and feline dermatitis and otitis.

Comparing the MIC values of staphylococci, *Pseudomonas aeruginosa* and *Streptococcus canis* against marbofloxacin and *Malassezia pachydermatis* against ketoconazole in 2010 and in 2018 we found no significant differences in MIC values.

MIC values examined show that staphylococci were highly sensitive to marbofloxacin. MIC₉₀, 0.25 µg/ml in 2010, (n=89), 1 µg/ml in 2017–2018, (n=135). The second sensitive bacterium was *Streptococcus canis*. MIC₉₀ 2 µg/ml in 2010, (n=43), 2 µg/ml in 2017–2018, (n=22). The less sensitive bacterium was *Pseudomonas aeruginosa* (MIC₉₀ 8 µg/ml in 2010, (n=56), 4 µg/ml in 2017–2018, (n=55).

M. pachydermatis was very sensitive to ketoconazole both in 2010 and 2017–2018 (MIC₉₀ 0.031 µg/ml in 2010, 0.063 µg/ml in 2017–2018).

According to our results it can be stated, that *M. pachydermatis* strains showed very high susceptibility to ketoconazole in their planktonic (suspended) forms. However, when planted on surface catheters to produce biofilms their susceptibility was changed significantly. These data highlighted that biofilm producing forms of a yeast strain can show 25–3125 times decrease in susceptibility (25–3125 times increase in MIC) compared to the planktonic forms of the respective strain.

2. Összefoglaló

Jelentős költségei miatt az utóbbi időben drasztikusan csökkent az új antibakteriális hatóanyagok fejlesztése világszerte. Ezzel egy időben, az állatgyógyászatban azon antibiotikumok használatát, amelyeket az human egészségügyben CIA vagy HIA-ként tartanak számon, korlátozzák vagy tiltják. Ezek a korlátozások és tiltások a jövőben még inkább előtérbe kerülnek, látva a mikrobiális rezisztencia fokozódását világszerte. Feltételezhető, hogy a meglévő antibakteriális hatóanyagok jól megválasztott kombinációiban számos szinergikus lehetőség maradt még rejtve jelenleg is, amelyek feltárása segíthet a bakteriális rezisztenciát terjedésének csökkentésében. *Escherichia coli* esetében már megerősítést nyert, hogy bizonyos antibiotikumok alkalmazása elősegítheti a baktérium érzékenységét más antibiotikummal szemben olyan esetekben is, amikor korábban az adott antibiotikummal szemben a baktérium hiper-rezisztenciát mutatott (Lázár *et al.*, 2013). Fontos továbbá a fix kombinációk tesztelése során, hogy az interakciók mellett megfigyelésre kerüljenek olyan tulajdonságok is, amelyek figyelembevételével növelhető a kombinációk biztonságos alkalmazása.

Jelen Értekezésben bemutatandó vizsgálatok elvégzésének célja kutyák külső hallójárat gyulladásának kezelésére számításba vehető néhány fix kombináció hatásának feltárása a kezelés hatékonyságának és megbízhatóságának javítása érdekében.

Eredményeink az alábbiakban összegezhetők:

Marbofloxacin és a gentamicin között szinergizmus került kimutatásra a vizsgált *P. aeruginosa* törzsek 48.5%-ában. Az átlagos FIC index 0,546, amely szinergiát, bár csak részleges szinergiát jelent a vizsgált törzsek átlagára nézve. A vizsgálatok alkalmával egyetlen törzs sem mutatott antagonista hatást. Az eredmények alapján a marbofloxacin–gentamicin kombináció indokolt és célzott alkalmazása a *P. aeruginosa* által okozott külső hallójárat gyulladások esetében előnyösnek tűnik. Különösen előnyös lehet az alkalmazás olyan esetekben, amikor a magasabb hatóanyag koncentráció használata, a hallójárat és dobhártya sérülése miatt kockázatos lenne.

IPEC-J2 egyrétegű sejtenyészeten történő penetrációt vizsgálva, a gentamicin rossz paracelluláris permeációt mutat. Az 1%-os DMSO hozzáadása nem szüntette meg a paracelluláris integritást, és nem segítette elő a gentamicin sejtrétegen történő átjutását. Eredményeink azt mutatják, hogy a DMSO ilyen alacsony koncentrációban a gyógyszerkészítmény részeként nem segíti elő a gentamicin hámszöveten keresztül történő felszívódását és így nem fokozza a gentamicin által okozott ototoxikus hatások előfordulását a fülcseppekben sem. A gentamicin alkalmazható továbbá az hámszövet sérülések, LPS-indukálta bakteriális fertőzés vagy akut oxidatív stressz okozta diszfunkciók kimutatására is, amikor az IPEC-J2 egyrétegű sejtenyészet által határolt bazolaterális terében megnövekszik a gentamicin koncentrációja a kontrollhoz képest. A HPLC módszerrel elvégzett vizsgálatunk az egyrétegű sejtenyészet integritását mutatta, mivel nem növekedett meg a vizsgálat során a bazolaterális térben a gentamicin koncentráció. Ez egyidejűleg elvégzett transzepiteliális elektromos rezisztencia (TEER) mérése is ezt támasztotta alá, mivel nem csökken az ellenállás a vizsgálat ideje alatt, aminek csökkenése a sejtréteg felbomlását mutatta volna.

8 napos sorozat-passzálás vizsgálat során a marbofloxacin és a marbofloxacin–gentamicin (1:1) kombináció minimális gátló koncentrációját (MIC) tizenhat *P. aeruginosa* törzsben vizsgáltuk. A szubletális koncentrációkban alkalmazott marbofloxacin oldatban a sorozatos passzálás szignifikánsan megemelte a naponként mért MIC-értékeket. Szubletális koncentrációkban a marbofloxacin–gentamicin (1:) MIC-értékek is növekedését mutattak, de ez a növekedés jóval alacsonyabb volt, mint a csak marbofloxacin oldatban mért érték. Egy törzs nem érte el a 32 µg/ml-nél magasabb MIC értéket.

A 8 napos sorozatpasszálást *S. pseudintermedius* baktériumokkal is végeztük. A MIC-ek átlagos növekedése a 8. napon a csak marbofloxacin oldatban tenyésztett törzseknél 11.67-szeres volt, szemben a marbofloxacin–gentamicin (1:1) kombinációban tenyésztett törzsek 3.00-szoros értékével. Ezenkívül megállapítható, hogy a kombináció 1. napi eredménye is általában alacsonyabb volt, így a MIC növekedés mértéke is jóval kisebb mértékű abszolút növekedést mutatott. Az eredmények mindegyike kifejezett szignifikáns különbséget mutatott ($p < 0,0001$). Figyelembe véve az eredményeket, kimondhatjuk, hogy a marbofloxacin: gentamicin (1: 1) kombináció jelentősen mértékben gátolhatja a *S. pseudintermedius* baktériumok fluorokinolonokkal szembeni rezisztenciájának kialakulását.

Összehasonlítottunk továbbá 2010-ben és 2017–2018 között, kutyák és macskák dermatitis és otitis externa klinikai eseteiből izolált *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Streptococcus canis* és *Malassezia pachydermatis* törzsek MIC értékeit. Sem az említett baktériumok marbofloxacinnal szemben, sem a *Malassezia pachydermatis* ketokonazollal szemben 2010-ben és 2017–2018 között mért MIC értékei között nem találtunk szignifikáns különbséget.

A vizsgált MIC értékek alapján a *Staphylococcus* spp. törzsek továbbra is nagyfokú érzékenységet mutattak marbofloxacinnal szemben. MIC₉₀, 0.25 µg/ml 2010-ben (n = 89), 1 µg/ml 2017–2018-ban (n=135). A második legérzékenyebb csoport a *Streptococcus canis*. volt. MIC₉₀ 2 µg/ml 2010-ben (n=43), 2 µg/ml 2017–2018-ban (n = 22). Kisebb érzékenységet mutatottak a *Pseudomonas aeruginosa* törzsek, de az érzékenység csökkenést ez a csoport sem mutatott. (MIC₉₀ 8 µg/ml 2010-ben (n=56), 4 µg/ml 2017–2018-ban (n=55).

A *Malassezia pachydermatis* 2010-ben és 2017–2018-ban is nagymértékben mutatott érzékenységet ketokonazollal szemben. MIC₉₀ 0.031 µg/ml 2010-ben (n=54), 0,063 µg/ml 2017–2018-ban (n=80).

Vizsgáltuk ezen felül 8 *Malassezia pachydermatis* törzs MIC értékeinek alakulását planktonikus és biofilm környezetben ketokonazollal szemben. Eredményeink alapján megállapítható, hogy a *Malassezia pachydermatis* törzsek nagymértékben érzékenyek ketokonazolra planktonikus állapotban. Ugyanezeknek a törzsek az érzékenysége viszont jelentős mértékben csökkent biofilm állapotban tenyésztve. A kapott MIC értékek alapján biofilmben a *Malassezia pachydermatis* érzékenysége 25–3125-szor volt kisebb a planktonikus állapotban szaporodó törzsekhez képest.

3. Introduction and objective

3.1. Introduction

Since the publication of the Swann report (Swann, 1969) it is clear, that the reason for resistance developed against antibiotics is multifactorial in which both veterinary and human medicine play an important role (Barton, 2000; Gustafson, 1997). In human healthcare, resistance developing during hospitalization (Feikin *et al.*, 2000; Niederman, 2001; Szűcs *et al.*, 2011) and due to the way patients apply antibiotics disregarding physician prescriptions (Llor and Bjerrum, 2014) has at least the same degree of significance. Several studies have been published in relation to the development of resistance in bacteria against antibiotics and its mechanisms (Malik *et al.*, 2005; Tenover, 2006). In order to decelerate the progress and keep it under control, the developed countries and federations of the world – such as the European Union (EMA/CVMP/209189/2015) – defined uniform principles. Among others, the professional aspects to be considered when applying antibiotics for veterinary purposes were also defined in the associated guidelines:

- newly developed antibiotic containing medications should have only one antibiotic agent, and be accompanied by precise indications
- an acceptably thorough justification is needed for the registration of a newly developed product that contains a fix combination
- the use of antibiotics should be targeted and preferably applied following susceptibility testing
- antibiotics should be applied exclusively under veterinary supervision.

The professional objective of my work is based on the hypothesis that inhibiting the growth of bacteria (and other microbes) and their effective elimination also depend on the number of points of the bacterial environment that are targeted by the damaging intervention. If the physical and/or chemical and/or biological parameters of the environment of a certain bacterium are changed from the physiological ones, then the combined effect of the factors damaging the bacterium is stronger and causes more permanent reduction in the number of bacteria than any scenario where only one parameter would be changed at a time. This phenomenon has been long recognized in the preserving industry. Applying temperature and pH value changes, ionizing

radiation, bacteriocin (nisin) and other chemical substances (carbon dioxide, organic acids) alone or in combination with the high-pressure treatment of food leads to a much more significant reduction of the number of bacteria than the same level high pressure treatment alone (Crawford *et al.*, 1996; Farkas *et al.*, 2003; Graells *et al.*, 1999; Kálmánné, 2009; Knorr, 1995; Paluo *et al.*, 1997; Papineau *et al.*, 1991; Popper and Knorr, 1990). A similar analogy can be assumed in cases where the elimination/inhibition of the bacteria is carried out with the use of antibacterial active agents that have their effects at two or more different targets. The more physiological aspects of the bacteria is damaged and/or the more intolerable their physiological environment is made, the more pronounced damaging effects can be expected. With regard to the EMA-CVMP guidelines on reducing bacterial resistance, We wish to add more refined distinctions to them by exploring further possibilities in my study.

In order to develop a model, We have selected bacteria causing skin inflammation in dogs and such antibacterial active agents used to treat bacterial skin inflammation that are applied locally (Dégi and Cristina, 2011). We have examined the effects of two antibacterial agents alone and in combination on a few important skin pathogen bacterial strains. We considered the following aspects when selecting the active agents:

- the target point of the two antibacterial agents should be essentially different
- the active agent should not or only minimally be absorbed through the skin
- they should be originally effective against the strains mainly causing skin inflammation in dogs and ¹cats:
 - *Staphylococcus pseudintermedius*,
 - *Staphylococcus aureus*, *Streptococcus canis*, *Pseudomonas aeruginosa* (Ganiere *et al.*, 2005; Mande and Kitaa, 2005; Morris *et al.*, 2006; Rosenkrantz, 2009; Sykes *et al.*, 2014)

In addition, the fact that Romanian researchers have already revealed certain synergistic interaction when enrofloxacin and gentamicin were applied simultaneously also played a role in choosing the active agent (Mărculescu *et al.*, 2007). However, We

¹ Having regard to the fact that no evidence is published of the difference in both phenotypic features and resistance profile between the target bacteria collected from canine and feline dermatitis or otitis cases we did not exculde aboved mentioned bacterium species isolated from cats. Our examinationas also support this presumption.

have chosen to use marbofloxacin instead of enrofloxacin in my study, firstly, because in the case of marbofloxacin general resistance supported by literary data is less frequent than with and secondly (Farca *et al.*, 2007; Pintarić *et al.*, 2017), because unlike in the case of enrofloxacin vision loss in cats has not been reported with marbofloxacin enrofloxacin (Gelatt *et al.*, 2001).

Based on the above-mentioned considerations, marbofloxacin and gentamicin have been selected as antibacterial agents for the experiments, and we tested their efficiency alone and in combination on *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, *Streptococcus canis* and *Pseudomonas aeruginosa* bacteria.

It is expected from an advanced medicinal combination intended to use on skin or in ear to be near pH neutral and free from pungent smell. For this reason DMSO is widely used in cases when active pharmaceutical ingredients cannot be solved in water based solution at neutral pH (e.g. prednisolone). On one hand DMSO seems to be a good solution in such cases, but on the other hand DMSO may facilitate the transmembrane transport of such antibiotic group like aminoglycoside that may cause ototoxic effect when absorbed in higher concentration through the cell membrane. In my experiments therefore, We would also like to get answer to the question that what effect the presence of DMSO may have on the transport mechanism through monolayer cells if used with gentamicin as a representative of aminoglycoside group (Dimethyl sulfoxide (DMSO) - American Chemical Society, Annual Meeting 2000).

Taking into consideration the fact that microbes can be found in biofilms both in case of inorganic materials and living organisations (Donlan and Costerton, 2002) we investigated the influence of biofilm on *Malassezia pachydermatis* MIC value too. *Malassezia pachydermatis* were selected for this examination due the fact that unlike bacteria there is a well-defined experimental method for fungi.

3.2. Objective

One aim of this study was to determine antimicrobial susceptibility of *P. aeruginosa* strains to marbofloxacin and gentamicin and investigate the possible synergistic, additive, indifferent or antagonistic effect between the two antibacterial agents with special emphasis on those strains that were resistant to antibiotic monotherapy.

We were also interested in determining the permeation of gentamicin across a monolayer of the porcine intestinal epithelial cell line, IPEC-J2 in the presence and absence of 1% DMSO using HPLC with fluorescence detection based on derivatization of the analyte with fluorenylmethyloxycarbonyl chloride (FMOC). This study was an *ex vivo* model to determine gentamicin permeation and consequent ototoxicity if applied in animals with intact tympanic membrane.

Another goal of this examination was to investigate the risk and process of resistance development of certain antibacterials. We investigated the development of resistance after 8-day serial passages in sublethal concentrations of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination in *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* strains isolated from external otitis cases in dogs.

Furthermore, we determined minimum inhibitory concentration (MIC) values of marbofloxacin and ketoconazole in selected *S. pseudintermedius*, *S. aureus*, *S. canis*, *P. aeruginosa* and *M. pachydermatis* strains isolated from dogs (from skin and the external ear canal). The first examinations were performed in 2010 and we repeated it with the same way in 2017–2018 to check the rate of resistance developed among the mentioned microbes in 2010 and 7–8 years later.

A further objective was to study minimum inhibitory concentration (MIC) values of ketoconazole in selected *Malassezia pachydermatis* strains isolated from dogs and grown on biofilms and as planktonic forms. The results of this study might help to predict *in vivo* efficacy of ketoconazole as yeasts often produce biofilms in different types of infections. The *in vivo* sensitivity of fungi can be much lower than determined during *in vitro* studies. The MIC-values were determined with the broth microdilution method in accordance with the guideline CLSI M07-A9. 2012. The biofilm production was achieved according to the method of Cannizzo *et al.* (2007) with slight modifications.

4. Literature review

In these days it is an increasing challenge to give appropriate and effective answers to emerging antimicrobial resistance. We believe that fix combinations of antibiotics have a suitable place in the arsenal of practitioners, and play an important role in fighting bacterial infections effectively. It is obviously not the Sorcerer's stone but it would be a mistake not to exploit their potentials.

According to the EU strategy on antimicrobials 2016–2020 CVMP in consensus with CHMP it is drastically restricted the use of antibiotics in veterinary sector. Three categories were established accordingly.

The AMEG has further categorised the WHO CIAs (and several HIAs) based on their risk to man due to resistance development following use in animals (EMA, 2014). The following three categories have been proposed by the AMEG:

- Category 1 contains the antimicrobials used in veterinary medicine where the risk for public health is estimated low or limited;
- Category 2 contains the antimicrobials used in veterinary medicine where the risk for public health is estimated as higher;
- Category 3 contains the antimicrobials not approved for use in veterinary medicine. (EMA and EFSA Joint Scientific Opinion, EFSA BIOHAZ Panel, 8 December 2016)

The most important antimicrobial classes in the WHO CIAs are categorised by AMEG as category 1 (macrolides), category 2 (fluoroquinolones, 3rd- and 4th-generation cephalosporins for systemic use) and category 3 (glycopeptides; not authorised in veterinary medicine in the EU). The extended spectrum penicillins and aminoglycosides are provisionally placed in category 2 while undergoing further risk-profiling. Polymyxins (colistin and polymyxin B) were originally classified by AMEG as category 1 but the classification of colistin was changed to category 2 after review in 2016 (EMA, 2016b, EMA and EFSA Joint Scientific Opinion, 2016). The initiation of this restriction was the increasing resistance of bacteria especially in human medicine. According to this “One Health” approach, development of sole active ingredient containing VMP is mainly preferred by EU. It does not mean that the use of fix

combinations is banned but for the registration of a fix combination, positive risk benefit balance should be adequately justified.

Given the current regulation and the fact that practically no new drug development in the field of veterinary medicine has been realized, the animal health sector needs to exploit the additional potential of existing antibiotics.

Combinations of fluoroquinolones with other antimicrobial agents have been extensively investigated.

Marbofloxacin

Marbofloxacin is a 3rd generation veterinary fluoroquinolone introduced in clinical practice at the end of the 20th century.

Fluoroquinolones as other quinolones act by converting their targets, gyrase and topoisomerase IV, into toxic enzymes that fragment the bacterial chromosome. This review describes the development of the quinolones as antibacterials, the structure and function of gyrase and topoisomerase IV, and the mechanistic basis for quinolone action against their enzyme targets (Aldred *et al.*, 2014). Fluoroquinolones have bactericidal and concentration-dependent effect (Hawkey 2003; McKinnon and Davis 2004).

Its broad antibacterial spectrum involves several Gram-negative bacteria, staphylococci and *Mycoplasma* spp. It has good activity against almost all bacterial species causing dermatitis in animals. Staphylococci and pasteurellae are highly sensitive, *P. aeruginosa* strains are usually susceptible, streptococci are moderately sensitive. Low incidence of resistance and excellent pharmacokinetic profile renders this antibiotic appropriate for use in veterinary dermatology. Local and systemic toxic effects after dermal absorption in animals are not yet reported. Ocular toxicity (retinal degeneration) reported at enrofloxacin could not be demonstrated in the case of marbofloxacin (Gelatt *et al.*, 2001).

CLSI defines MIC values of 1 µg/ml and lower as susceptible, 2 µg/ml as intermediately resistant and 4 µg/ml and higher as resistant. It is investigated the antibacterial effect of marbofloxacin against 284 staphylococcal, 64 streptococcal and 30 *P. aeruginosa* strains. High susceptibility and low minimum inhibitory concentrations (MIC) were

reported in staphylococci (*S. pseudintermedius*, *S. aureus* and coagulase negative staphylococci, e.g. *S. epidermidis*) with MIC₅₀ and MIC₉₀ values as 0.19 and 0.38, respectively (Spreng *et al.*, 1995). Higher MIC values were experienced in the case of streptococci with MIC₅₀ and MIC₉₀ values of 1.7 and 3.8, respectively. These data define streptococci as intermediately resistant Gram-positive bacteria to marbofloxacin. Moderately low MIC values were noticed in *P. aeruginosa* strains with MIC₅₀ and MIC₉₀ values of 0.43 and 0.94, respectively. Bactericidal activity of marbofloxacin is similar to enrofloxacin in a time-dependent manner. Minimum bactericidal concentrations (MBC) are 2–4 fold of MICs. Significant decrease in bacterial population of staphylococci was observed after 3 hours. Post antibiotic effect (PAE) of marbofloxacin proved to be 2.4 hours against *S. pseudintermedius*.

High susceptibility in staphylococci to marbofloxacin with MIC values ranging from 0.12 to 1 has been reported. No significant change in MICs was observed between strains isolated in 1999, 2000 and 2001. *P. aeruginosa* strains also proved to be sensitive, the three year survey found 57 sensitive and 5 resistant strains isolated from the skin, and 51 sensitive and 9 resistant strains isolated from otitis externa cases in dogs and cats. Streptococci are usually moderately sensitive, however, a 2004 survey indicated high susceptibility of streptococci against the substance (Meunier *et al.*, 2004).

P. aeruginosa is an important veterinary pathogen against which fluoroquinolones are commonly used. It was examined 10 clinical isolates (obtained from the North Carolina State University Veterinary Teaching Hospital and the Rollins Animal Disease Diagnostic Laboratory) and tested the MIC. Antimicrobials were tested in the following ranges: ciprofloxacin, 0.157 to 5.0 mg/ml; enrofloxacin, 1.25 to 5.0 mg/ml; marbofloxacin, 0.625 to 5.0 mg/ml; orbifloxacin, 0.625 to 5.0 mg/ml. The results presented here show that ciprofloxacin does not provide an adequate representative for assessing the *in vitro* susceptibility of fluoroquinolones used in veterinary medicine. It was also found no correlation between ciprofloxacin susceptibility and that of the other three fluoroquinolones when using clinical isolates of *P. aeruginosa*, suggesting that, at least for this organism, ciprofloxacin does not provide an acceptable alternative for susceptibility testing (Riddle *et al.*, 2000).

In another experiment in Europe, where samples were taken from bovine mastitis and respiratory disease, 99.30 per cent of *S. aureus* isolates were susceptible to marbofloxacin. MIC for these mastitis strains were all centred on 0.25 µg/ml,

constituting a single population. Three isolates in 2002 and one in 2005 were intermediate (MIC=2 to 4 µg/ml) and resistant (MIC=32 µg/ml), respectively. MIC₅₀ and MIC₉₀ did not show any evolution since 2004. Coagulase-negative staphylococci strains were not targeted by the study before 2004, hence the data were only based on a five-year period. Results for coagulase-negative staphylococci isolated from bovine mastitis were very similar to those obtained for *S. aureus*, but MIC₉₀ was a dilution step higher. Between 2004 and 2008, MIC₅₀ and MIC₉₀ did not show any significant evolution. Overall, 99.29 per cent of the tested coagulase-negative staphylococci strains were susceptible to marbofloxacin over the five-year period (Kroemer *et al.*, 2012).

Marbofloxacin has been licensed for use in various mammalian species, but not as yet for turkeys, although its kinetic properties distinguish it from other fluoroquinolones. The longer half-life of marbofloxacin in many animal species has been appreciated in veterinary practice. It is generally accepted that, for fluoroquinolones, the optimal dose should be estimated on the basis of the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics of the drug under consideration. Knowledge of these specific data for the target animal species allows the establishment of an integrated PK-PD model that is of high predictive value. In the present study, the antibacterial efficacy (PD indices) against a field isolate of *Escherichia coli* O78/K80 was investigated *ex vivo* following oral and intravenous administration of marbofloxacin to turkeys (breed BUT 9; six animals per group) at a dose of 2 mg/kg of body weight (BW). These first results suggested that the recommended dose of 2 mg/kg BW of marbofloxacin is sufficient to achieve a therapeutic effect in diseased animals. However, considering the risk of resistance induction, the applied dose should be equal to an AUC/MIC of >125, the generally recommended dose for all fluoroquinolones. According to the PK-PD results presented here, a dose of 3.0 to 12.0 mg/kg BW per day would be needed to meet this criterion. In conclusion, the results of the present study provide the rationale for an optimal dose regimen for marbofloxacin in turkeys and hence should form the basis for dose selection in forthcoming clinical trials (Haritova *et al.*, 2006).

It is also reported 91.3% incidence of susceptibility to marbofloxacin in *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs (Barrasa *et al.*, 2000). Only 52.1% of the strains were sensitive to enrofloxacin however, probably because of its indiscriminate use (Šeol *et al.*, 2002) investigated 183 *P. aeruginosa* strains isolated

from the outer ear canal of dogs, 93.4% was sensitive to marbofloxacin. In another experiment a total of 194 staphylococcus isolates from 310 dogs were tested (Vanni *et al.*, 2009). Most of the strains proved to be *S. pseudintermedius* and some *S. schleiferi*. 98.2% of the *S. pseudintermedius* strains were highly susceptible to marbofloxacin.

Gentamicin

Aminoglycoside antibiotics inhibit protein synthesis and induce a significant increase in misreading of messenger RNA, which is highly dependent on the antibiotic concentration. They are also known to inhibit ribosomal translocation and to stabilize the EFG-GDP-ribosome complexes. Experiments performed on individual steps of protein synthesis indicate that their binding on 30S subunits could induce misreading, while their binding on a 50S subunit could induce the stabilization of the complexes; inhibition of translocation could possibly be due to a simultaneous fixation on both subunits (Tangy *et al.*, 1985). Gentamicin acts bactericidal and concentration-dependent way (McKinnon and Davis 2004).

Gentamicin is one of the most potent aminoglycosides used in the veterinary medicine. The antibacterial spectrum involves mainly Gram-negative aerobic bacteria and staphylococci. It has good activity against almost all bacterial species causing otitis in animals. Staphylococci are highly sensitive, *P. aeruginosa* strains are usually susceptible. Concentration-dependent quick bactericidal effect and relatively bad absorption after topical administration (because of hydrophilic nature) makes it appropriate for safe usage in veterinary dermatological cases. Ototoxicity, however, lays claim to intact tympanic membranes if applied locally into to external ear canal. Transcellular penetration of gentamicin is not possible according to its highly polar and hydrophilic nature. Paracellular penetration across tight junctions, however, might take place across the cell monolayers. DMSO as a lipophilic organic solvent that might be able to enhance this process.

It was reported 65.2% incidence of susceptibility to gentamicin in *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs (Barrasa *et al.*, 2000). According to another investigation 183 *P. aeruginosa* strains isolated from the outer ear canal of dogs, 83.1% of the strains were susceptible to gentamicin (Šeol *et al.*, 2002).

Experienced in a study in 1979, 75% susceptibility to gentamicin among *P. aeruginosa* strains isolated from animals (Hirsh *et al.*, 1979).

Synergy between gentamicin and amoxicillin against mutant *Enterococcus faecalis* strains is described. All mutants were stable after four subcultures on BHI agar. Gentamicin alone at one-half, one-quarter, one-eighth, and 1/16th the MIC for all *E. faecalis* strains was ineffective. Amoxicillin alone at a concentration of one-half the MIC was not bactericidal against any of the strains studied. The amoxicillin concentration used was 0.25 µg/ml (one-half the MIC) because no synergism with gentamicin was detectable with an amoxicillin concentration above or equal to the MIC (0.5 µg/ml). The amoxicillin and gentamicin combination was bactericidal and synergistic against *E. faecalis* even at low gentamicin concentrations (4 µg/ml: 1/16th the MIC). The gentamicin concentrations required to observe bactericidal activity and synergism in combination with amoxicillin increased from 1/16th to one-eighth, one-quarter, and one-half of the MIC for strains respectively (Aslangul *et al.*, 2005).

High resistance to aminoglycoside among also *Enterococcus* spp. is also described. The strains were defined as *Enterococcus faecium*, although of the VSE strains, 53% were identified to be as *Enterococcus faecalis*, 42% *E. faecium*, 3% *Enterococcus durans*, and 2% *Enterococcus avium*. High-level resistance to vancomycin (MIC >256 µg/ml) was determined in all VRE strains and when analyzing MIC values for teicoplanin, five strains were found to be moderately susceptible (MIC, 16 µg/ml) and 95 strains were resistant (MIC, >32 µg/ml). Of the VRE strains, one was linezolid-resistant (MIC 12 µg/ml) and the other was intermediately susceptible (MIC, 4 µg/ml) and remainders were evaluated to be susceptible (MIC, <2 µg/ml). In VRE strains, high-level gentamicin resistance (HLGR) was found to be 83% and high-level streptomycin resistance (HLSR) 89%, association of HLSR with HLGR was 78%. In VSE strains, HLGR was found to be 42% and, HLSR 48%, the association of HLSR with HLGR was found to be 36%. HLAR in VRE strains was found to be higher as compared with VSE strains (p <0.005) (Baldir *et al.*, 2013).

New gentamicin resistance genes were also discovered. With the report of the new aminoglycoside resistance genes *aph(200)-Ic*, *aph(200)-Id*, and most recently, *aph(200)-Ib*, *aac(60)-Ie-aph(200)-Ia* is no longer the only aminoglycoside resistance gene in enterococci known to encode resistance to gentamicin, and a new approach to detecting resistance to aminoglycoside synergism may be required. The *aph(200)-*

lc gene was initially isolated from an *Enterococcus gallinarum* animal isolate but was subsequently also found in *E. faecium* and *Enterococcus faecalis* clinical isolates. Although the gentamicin MIC is 256–384 mg/ml for *enterococci* that possess *aph(200)-lc*, these isolates are nonetheless resistant to ampicillin-gentamicin synergism (Chow, 2000).

Antibacterial susceptibility against gentamicin was confirmed by studying inhibitory zone diameter on agar solid media at MIC level of these compounds against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus*. In this experiment gentamicin and azithromycin shows very significantly bigger zone diameter against all organisms followed by vancomycin & chloramphenicol and then cefotaxime. From these observations azithromycin can be chosen as susceptible antibacterial agent against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus*. Though gentamicin shows MIC at higher concentration than vancomycin, chloramphenicol and cefotaxime but the compound exerts significant bactericidal effect as well in that concentration (Das *et al.*, 2014).

Gentamicin susceptibility in *E. coli* was also reported. 120 *Escherichia coli* isolates positive for one of the gentamicin resistance (GEN^R) genes *aac(3)-II*, *aac(3)-IV* or *ant(2'')-I* were tested for gentamicin susceptibility by the agar dilution method. Isolates positive for *aac(3)-IV* or *ant(2'')-I* had an MIC distribution of 8–64 mg/l, whereas isolates positive for *aac(3)-II* had MICs of 32 to >512 mg/l, suggesting a relationship between the distribution of MICs and the specific GEN^R mechanism. The MIC distribution, regardless of the GEN^R mechanism, was 8 - >512 mg/l (Jakobsen *et al.*, 2007).

Gentamicin can be successfully used against *Neisseria gonorrhoeae*. Sentinel surveillance of gentamicin susceptibility showed that 95% of European isolates were within a narrow MIC range (4–8 mg/l), with 79% showing an MIC of 8 mg/l. Most countries showed little variation, but wider MIC ranges were observed in Greece (1–16 mg/l) and France, Norway and Sweden (2–16 mg/l) (Chisholm *et al.*, 2010).

It is also reported, that despite the relatively high prevalence of multidrug-resistant coagulase-positive *staphylococci*, isolated from dogs with otitis externa in Trinidad, are largely susceptible to gentamicin (83.1%, n=114) consistent with use in clinical practice. The first detection of methicillin-resistant *S. pseudintermedius* (MRSP) in dogs is likely to have implications on the treatment options for otitis externa in dogs and potential public health significance (Dziva *et al.*, 2015).

Ketoconazole

Ketoconazole is one of the most potent imidazole antifungal drugs with broad antifungal spectrum and wide margin of safety. Compared to other imidazoles (miconazole, enilconazole, clotrimazole) it has higher activity, resistance is less common and its spectrum is broader. Antifungal spectrum involves yeasts and dermatophytes alike. It has excellent activity against almost all fungal species causing dermatitis in animals. *Malassezia* spp., *Candida albicans*, *Trichophyton* spp. and *Microsporum* spp. are usually highly sensitive (Hector, 2005). Good pharmacokinetic profile and resistance pattern renders it appropriate also for local and/or systemic usage in veterinary dermatological cases. The fungistatic effect correlated with inhibition of ergosterol synthesis and elevated lanosterol/ergosterol ratios in the organisms. The fungicidal effect involved rapid membrane damage and was unrelated to the imidazole-induced block in ergosterol synthesis (Sud *et al.*, 1981).

M. pachydermatis colonizes the skin and mucosal sites of healthy dogs and cats. Favorable growth conditions in the local environment allow excessive multiplication of this organism, which may then function as an opportunistic secondary pathogen. Malassezia dermatitis and otitis, inflammatory diseases associated with elevated populations of *M. pachydermatis* on the skin and in the ear canal of dogs and cats, have been recognized with increasing frequency. The underlying conditions leading to the yeast overgrowth include hypersensitivity diseases (atopy, adverse cutaneous food reactions, flea bite hypersensitivity, and contact allergy), cornification disorders, ectoparasite infection, bacterial pyoderma, and endocrine diseases (hyperadrenocorticism, hypothyroidism, diabetes mellitus). Moreover, a hypersensitivity response to the yeast itself is likely to occur in many allergic dogs. Current treatment options for Malassezia dermatitis/otitis in dogs include systemic and/or topical therapy with a number of antifungal agents, in addition to various antiseptics (Peano *et al.*, 2017).

Antifungal susceptibility testing of *Malassezia* species remains a challenge, as their growth is not supported (or, in the case of *M. pachydermatis*, only poorly supported) on the standard lipid-free RPMI growth medium recommended for yeast testing by the CLSI and EUCAST. A variety of alternative procedures, most of which use lipid-enriched media, have been proposed, but published studies have mainly focused on *M. pachydermatis* susceptibility to ketoconazole, itraconazole, and other azole

derivatives. On the contrary, information on the *in vitro* susceptibility of *M. pachydermatis* isolates to the polyene amphotericin B, which is commonly used for treating bloodstream infections in human patients, is scarcer (Álvarez-Pérez *et al.*, 2014).

It can be concluded that ketoconazole is highly active against *M. pachydermatis* strains (Lorenzini *et al.*, 1985). Canadian researcher stated that human isolates of malasseziae are highly susceptible to ketoconazole having a MIC range between 0.03 and 0.125 µg/ml (Gupta *et al.*, 2000).

The drug has also good activity against dermatophytes hence appropriate for the treatment of ringworm infections. It's been tested the clinical effectiveness of ketoconazole against *Microsporum* spp. infections in dogs and cats with 96.8% and 90.5% clinical cure in cats and dogs, respectively (De Keyser and Van den Brande, 1983). According to another experiment 12 cats having dermatophytosis with ketoconazole were treated (Medleau and Chalmers, 1992). Eight cats showed clinical cure after a median 6 week long treatment with 10 mg/kg of ketoconazole. Infection in one cat reacted only to double dosage.

Malassezia pachydermatis often occurs in biofilm. *Malassezia pachydermatis* fungemia has been reported in patients receiving parenteral nutrition. Biofilm formation on catheters may be related to the pathogenesis of this mycosis. It was investigated the biofilm-forming ability of 12 *M. pachydermatis* strains using a metabolic activity plate-based model and electronic microscopic evaluation of catheter surfaces. All *M. pachydermatis* strains developed biofilms but biofilm formation showed variability among the different strains unrelated to their clinical origin. This study demonstrates the ability of *M. pachydermatis* to adhere to and form biofilms on the surfaces of different materials, such as polystyrene and polyurethane (Cannizzo *et al.*, 2007).

Malassezia pachydermatis and *Candida parapsilosis* are often coisolated in case of canine seborrhea dermatitis (SD) and also are emerging as opportunistic pathogens of immunocompromised human beings. Increased information about how their relationship results in biofilm production and an antifungal response would be useful to inform treatment and control. It was demonstrated that regardless of yeast strain or origin all single and dual cultures produced biofilms within 24 hours, and the greatest amount was present after 72 hours. Biofilm production from mixed cultures was greater

than for single strains ($P < .05$). All sessile forms of the single and dual cultures were resistant to the tested antifungals itraconazole and ketoconazole, whereas planktonic forms were susceptible. It is suggested, that dual cultures produce stronger biofilms that are likely to enhance persistence in skin lesions in dogs and result in greater resistance to antifungal treatment (Bumroongthai et al., 2016).

It is published, that *M. pachydermatis* biofilm formation is associated with antifungal resistance, paving the way towards investigating drug resistance mechanisms in *Malassezia* spp (Figueredo et al., 2013).

According to the CLSI recommendations, *Malassezia pachydermatis* MIC values of 0.125 µg/ml and lower are defined as susceptible, 0.25–0.5 µg/ml as intermediately resistant and 1 µg/ml and higher as resistant. Researchers evaluated the antifungal effects of ketoconazole, itraconazole and fluconazole against 44 *M. pachydermatis* strains isolated from the skin and external ear canal of dogs. 70.8% of the isolates were susceptible, 12.5% moderately susceptible and 16.7% resistant to ketoconazole. Mean MIC was 0.70 µg/ml to ketoconazole with the broth microdilution method (Da Silva Nascente et al., 2003).

Malassezia pachydermatis is associated with dermatomycoses and otomycosis in dogs and cats. It is compared the susceptibility of *M. pachydermatis* isolates from sick and healthy animals to azole and polyene antifungals. Isolates from healthy animals were less sensitive to amphotericin B, nystatin, fluconazole, clotrimazole and miconazole (Weiler et al., 2013).

In another experiment, all *M. pachydermatis* isolates (n=45) were susceptible to itraconazole, ketoconazole, nystatin and terbinafine but resistant to 5-fluorocytosine. By Broth Microdilution Method, over 95% of *M. pachydermatis* isolates were susceptible to ketoconazole and itraconazole with an MIC₉₀ < 0.03 and 0.12 µg/ml, respectively (Yurayart et al., 2013).

According to another publication about 94% of *Malassezia* strains might be categorized within susceptible population for all azoles, except for fluconazole, and azole cross-resistance was detected in association with fluconazole in *M. pachydermatis* but not in *M. furfur* (Cafarchia et al., 2015).

4.1. Effect of the combination of marbofloxacin and gentamicin on the antimicrobial susceptibility of *Pseudomonas aeruginosa* strains

Pseudomonas aeruginosa is a frequently isolated Gram-negative bacterium in veterinary practice, associated mainly with external otitis, deep pyoderma, keratoconjunctivitis, respiratory and urinary tract infections (Kroemer *et al.*, 2013; Meunier *et al.*, 2004). This bacterium can develop resistance rapidly against several antibiotics, even during the course of antimicrobial treatment (Mouton *et al.*, 1999; Chastre *et al.*, 2003). Pandrug-resistant strains not susceptible to any of these substances have also been reported in the human medicine (Souli *et al.*, 2008). Accordingly, synergistic combinations of antibiotics are used frequently to eliminate this pathogen in human and veterinary practice alike. Synergism between enrofloxacin and gentamicin in 5 *P. aeruginosa* strains is scientifically presented (Anca *et al.*, 2007). Combinations of fluoroquinolones with aminoglycosides, beta-lactams, imidazoles, macrolides and clindamycin infrequently but also show synergy against the members of family Enterobacteriaceae and Gram-positive bacteria (Neu, 1991).

Marbofloxacin is a second generation fluoroquinolone with marked efficacy against *P. aeruginosa*. It has better activity (Farca *et al.*, 2007; Müller and Horn, 2009; Kroemer *et al.*, 2013) and a longer postantibiotic effect against the pathogen compared to enrofloxacin (Carbone *et al.*, 2001). A 91.3% incidence of sensitivity to marbofloxacin in *Pseudomonas* spp. isolated from dogs had been reported (Martin Barrasa *et al.*, 2000). 183 *P. aeruginosa* strains isolated from the outer ear canal of dogs were investigated (Šeol *et al.*, 2002) and found that 93.4% were sensitive to marbofloxacin. 26.1% resistance ratio in *P. aeruginosa* strains isolated from otitis cases in dogs and cats were reported in 2004 (Meunier *et al.*, 2004). According to another publication it was indicated 23% resistance and 16% moderate susceptibility ratio among 56 isolates (Jerzsele *et al.*, 2013). These data show an increasing tendency of marbofloxacin resistance among *P. aeruginosa* strains in companion animals.

Gentamicin is an aminoglycoside frequently used in veterinary medicine. It is applied as a topical medication in dermatology or given parenterally for respiratory, urinary or systemic *P. aeruginosa* infections. 65.2% incidence of susceptibility to gentamicin in *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs were published (Martin Barrasa *et al.*, 2000). According to another publication 183 *P. aeruginosa*

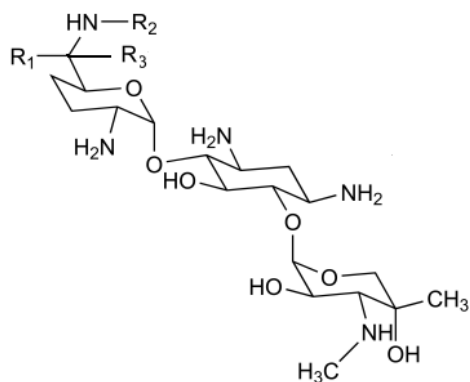
strains isolated from the outer ear canal of dogs; 83.1% of the strains were susceptible to gentamicin (Šeol *et al.*, 2002). Similar results were reported by Pedersen *et al.* in 2007, 84.6% of the investigated 39 *P. aeruginosa* strains were sensitive to gentamicin.

4.2. Penetration of gentamicin and gentamicin supplemented with 1% DMSO across biological monolayer membranes *in vitro*

Gentamicin is one of the most potent aminoglycoside antibiotics used frequently in the veterinary and human medicine for treatment of infections caused mainly by Gram-negative aerobic bacteria and staphylococci. Under certain physiological and pathological conditions, however, its systemic use is ototoxic and nephrotoxic, thus it cannot be administered to young or dehydrated animals and to patients with renal insufficiency. In the companion animal practice gentamicin is often administered topically such as suspensions, creams, ointments or ear drops, if the tympanic membrane is still intact (Parravicini *et al.*, 1982; Oghan *et al.*, 2011). Direct contact with the inner ear at high local concentrations can result in severe irreversible ototoxic effects (El Bakri *et al.*, 1998). A higher incidence of ototoxicity was reported in animals than humans (Morizono, 1988) following topical administration as an ear drop.

Gentamicin can also cause systemic toxic effects if it is absorbed through biological membranes, like the tympanic membrane, conjunctiva or intestinal epithelium. Although oral bioavailability of gentamicin is usually less than 3% in healthy animals, oral uptake might also be a toxicological issue if topical preparations containing surfactant, dimethyl sulphoxide (DMSO) are consumed and ingested by animals adding that even 10% of DMSO concentration did not have an effect on the permeability of the apical membrane or tight junctional complexes in Caco-2/TC7 cells (Da Violante *et al.*, (2002). Therefore, it is important to determine if DMSO supplementation increases the transport of gentamicin across cell layers, which would increase the potential toxic effects of the aminoglycoside.

Structurally, gentamicin is a 4,6-disubstituted aminocyclitol composed of the core aminocyclitol moiety, 2-deoxystreptamine (2-DOS), which is complemented with purpurosamine and garosamine aminosugars at positions C-4 and C-6, respectively (Figure 1).



Gentamicin C ₁	R ₁ =R ₂ =CH ₃	R ₃ =H
Gentamicin C _{1a}	R ₁ =R ₂ =R ₃ =H	
Gentamicin C _{2a}	R ₁ =R ₂ =H	R ₃ =CH ₃
Gentamicin C ₂	R ₁ =CH ₃	R ₂ =R ₃ =H

Figure 1. Chemical structure of gentamicin complex: C₁, C_{1a}, C₂ and C_{2a}.

In its therapeutic form, gentamicin comprises a complex of gentamicin C₁, C_{1a}, and C₂, and they differ only in the degree of methylation of the C-6' position of the sugar attached at C-4 of 2-DOS (Testa and Tilley, 1976). Other component of gentamicin is C_{2a}, which is a 6'-C epimer of C₂ (Seidl and Nerad, 1988). Several papers on high-performance liquid chromatographic methods (Isoherranen *et al.*, 2000; Al-Amoud *et al.*, 2002) with fluorometric detection have been published for quantitative determination of gentamicin complex consisting closely related compounds such as C₁, C_{1a}, C₂ and C_{2a}. Among derivatization techniques, o-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) are frequently applied to detect gentamicin in complex biological matrices. In our model system, IPEC-J2 cell line was grown on microporous membranes to ensure spontaneous cell differentiation, which is prerequisite for studying the transport processes through polarised epithelial monolayer present between apical and basolateral compartments (Tremblay *et al.*, 2006, Langerholc *et al.*, 2011, Cencic *et al.*, 2010).

Transcellular permeation of gentamicin is practically not possible in view of its relatively high molecular weight, highly polar and hydrophilic nature (Rama Prasad *et al.*, 2003). Paracellular permeation across tight junctions, however, might occur (González-Mariscal *et al.*, 2003, Madara *et al.*, 1989) and DMSO can facilitate this process by modulating protein assembly in tight junctions. At higher concentrations, DMSO is known to disrupt lipid structures in epithelia (Gordeliy *et al.*, 1998 and Simons, 2008) and thus it can facilitate penetration of certain substances. It was previously described that labrasol, a non-ionic surfactant, enhanced the oral bioavailability of gentamicin (Hu *et al.*, 2001) probably via tight junction opening mechanisms.

4.3. Effect of 8-day serial passage on the development of resistance against marbofloxacin–gentamicin (1:1) combination in *Pseudomonas aeruginosa* and *Staphylococcus pseudintermedius* strains

The ever-challenging increase in bacterial resistance poses a major global public health threat in humans and animal populations (Beco *et al.*, 2013; El Zowalaty *et al.*, 2012; Kroemer *et al.*, 2014, Niculae *et al.*, 2009; Rubin *et al.*, 2008). The increasing frequency of multidrug resistant (MDR) bacterial strains all over the world is particularly worrisome (Cristina and Degi 2013; Futagawa-Saito *et al.*, 2007). Besides the existing therapeutic tools, alternative solutions need to be developed and implemented based on the revised strategy against bacterial resistance (Golkar *et al.*, 2014; Karthik *et al.*, 2014; Stanton, 2013). One of the tools in this process is the use of antibiotic combinations. Careful application of combinations of different antibiotics having different mechanisms of action could be an effective therapy in several cases of bacterial infections (Ahmed *et al.*, 2014). The improper use of antibiotics (especially sub inhibitory or inadequate antibiotic therapy) contributes to the increased occurrence of antimicrobial resistance (Hughes *et al.*, 2014; Isturiz, 2010). It is also important to mention that the occurrence of bacterial resistance during monotherapy had already been described between 1985 and 2000 (Milatovic and Braveny, 1987; Mouton, 1999). Feline and canine animals among other pet species may serve as a potential reservoir of antimicrobial resistant bacterial isolates (Guardabassi *et al.*, 2004), and play an important role in the spill over of resistant bacterial isolates into humans. *Pseudomonas*

aeruginosa is a leading environmental opportunistic pathogen causing serious infections in humans and animals including wild, pet (dogs and cats), and domestic livestock (cattle, poultry and ostriches). The bacterium causes different skin and ear infections in canine and feline animals (Hariharan *et al.*, 1995; Šeol *et al.*, 2002; Hillier *et al.*, 2006).

Fluoroquinolones (FQs) are among the most widely used antimicrobial agents for treatment of veterinary pathogens including *Pseudomonas aeruginosa* infections (Martinez, 2006). Their dose regimens, pharmacokinetic concentrations, and minimum preventive concentrations require more understanding to guard against their selective pressure on the selection of resistant mutants. Marbofloxacin is widely used in veterinary medicine for treatment of feline and canine infections (Spreng *et al.*, 1995) and the choice of marbofloxacin and gentamicin combination in the present study was based on the observed synergistic interactions between fluoroquinolones and aminoglycosides (Jerzsele and Pászti-Gere, 2015; Mărculescu *et al.*, 2007). The present study aimed to investigate the degree of change in the minimum inhibitory concentrations (MICs) if the tested *P. aeruginosa* strains were separately cultured at sublethal concentrations of marbofloxacin alone and a combination of marbofloxacin and gentamicin at 1 to 1 ratio. *Pseudomonas aeruginosa* was selected as a target in this experiment because this species has high mutability and the genus is one of the most diverse bacterial genera known (Conibear *et al.*, 2009; Spiers *et al.*, 2000).

Staphylococcus pseudintermedius was also selected to be target bacterium species in this experiment based on the following aspects:

- *Staphylococcus pseudintermedius* very often occurs in feline and canine otitis externa (Hariharan *et al.*, 2014; De Martion *et al.*, 2016).
- We wonder if there is any difference in the results of the examination carried out with a Gram-positive (*Staphylococcus aureus*) and a Gram-negative (*Pseudomonas aeruginosa*) bacterium.
- *Staphylococcus pseudintermedius* can be considered as sensitive bacterium to marbofloxacin (Kroemer *et al.*, 2013).
- *Staphylococcus pseudintermedius* is highly capable of developing mutations (McCarthy *et al.*, 2015).

4.4. Susceptibility of *Staphylococcus* spp., *Streptococcus canis* and *Pseudomonas aeruginosa* strains to marbofloxacin and susceptibility determination of *Malassezia pachydermatis* strains to ketoconazole

MIC is the concentration at which no bacterial growth is observed. The lack of growth at MIC concentration may be caused by either bacterial killing or static effect on the examined bacterium. (Hansen and Blondeau, 2005).

MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints. Standardized methods for determining MICs is described in this paper. Like all standardized procedures, the method must be adhered to and may not be adapted by the user. The method gives information on the storage of standard antibiotic powder, preparation of stock antibiotic solutions, media, preparation of inoculum, incubation conditions, and reading and interpretation of results. Tables giving expected MIC ranges for control NCTC and ATCC strains are also supplied (Andrews, 2001).

Historically, the MIC has been the major PD marker used in guiding the dosing of antibacterial drugs. Earlier, for all antibiotics, the dose was selected so that the plasma concentration of the drug exceeded the MIC for as long as possible. In the last decades, studies using *in vitro* and animal models have played an essential role in reaching a more detailed understanding regarding the relationship between the PK and PD properties of antibacterial agents (Andrews, 2001).

The PD parameters, such as MIC, describe the inhibitory effect of the drug on bacterial pathogens. The three most frequently used PK/PD indices are ratio of area under the concentration time curve at 24 h to the MIC (AUC_{0-24}/MIC), the ratio of the peak drug concentration to the MIC (C_{max}/MIC), and the percentage of the dosing interval for which the plasma concentration exceeds the MIC ($\%T > MIC$). Using of PK/PD approach in veterinary medicine can optimize the right dosing regimen (Somogyi *et al.*, 2018).

The following data show that minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of marbofloxacin against *Enterobacter* spp., *Klebsiella* spp., *P. aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas* spp.,

Staphylococcus aureus and *Staphylococcus* spp. isolated in Thailand were 6.25, 3.45, 2.92, 1.93, 1.63, 8.32, 13.30, 6.77 (MIC) and 13.28, 6.32, 6.47, 3.53, 3.65, 17.23, 24.68, 17.06 (MBC) µg/ml, respectively (Chatchawanchonteera *et al.*, 2010).

In another experiment conducted in Canada the modal MIC results for ciprofloxacin, levofloxacin and garenoxacin against the *E. coli*, *C. freundii*, *E. cloacae* and *K. pneumoniae* strains were <0.06 µg/ml, <0.06 µg/ml, <0.25 µg/ml and <0.06–0.125 µg/ml respectively and modal MPC results were similar; modal MPC results respectively ranged between 0.125–0.5 µg/ml, 0.25–1 µg/ml and 0.25–4 µg/ml; modal MIC and MBC results were higher against the *P. aeruginosa* strains being <0.06–0.25 µg/ml, 1–2 µg/ml and 2–4 µg/ml respectively for ciprofloxacin, levofloxacin and garenoxacin (Hansen and Blondeau, 2005).

The Minimum Fungicidal Concentration (MFC) is similar to the MBC regarding to their principle. MFC is equal or higher than MIC depending on the antifungal active ingredient.

In this publication with regard to the antifungal susceptibility test (MIC), the presented results show that ketoconazole was highly effective against 40 isolates of the fungus with MIC ranging between 0.03–0.5 µg/ml. MIC₅₀ was 0.06 µg/ml and MIC₉₀ was 0.25 µg/ml. However, drug acts as fungicidal action (MFC) in 19 (47.5%) of isolates and fungistatic action (MIC) in 21 (52.5%) of isolates (Hessen, 2007).

Methods for assessing *in vitro* bactericidal activity have been well described (Clinical and Laboratory Standard Institute – CLSI M07-A9. 2012; Nascente *et al.*, 2009).

4.5. Activity of ketoconazole against biofilm producing and planktonic *Malassezia pachydermatis* strains

Ketoconazole is one of the most potent imidazole antifungal drugs with broad antifungal spectrum and wide margin of safety. Compared to other imidazoles (miconazole, enilconazole, clotrimazole) it has better activity, resistance is less common and its spectrum is broader. Antifungal spectrum involves yeasts and dermatophytes alike. It has excellent activity against almost all fungal species causing dermatitis in animals. *Malassezia* spp., *Candida albicans*, *Trichophyton* spp. and *Microsporum* spp. are usually highly sensitive (De Keyser *et al.*, 1983; Hector, 2005).

Malassezia pachydermatis strains of animal origin are usually highly susceptible to the azoles, being most sensitive to itraconazole and ketoconazole (Jesus *et al.*, 2011). Even fluconazole resistant strains of the yeast can be susceptible for ketoconazole (Jesus *et al.*, 2011). Good pharmacokinetic profile and resistance pattern renders it appropriate also for local and/or systemic usage in veterinary dermatological cases.

It was highlighted however, that some yeasts (especially *Candida albicans*) can produce biofilms *in vivo*, that results in a 30–2000 times decrease in sensitivity to certain antifungals including ketoconazole (Martinez *et al.*, 2010; Vandeputte *et al.*, 2012) due to the fact, that biofilm provides fungi defense against ketoconazole in a way, that biofilm hinders ketoconazole to reach cells of fungi and penetrate into them. It is not clarified that other yeasts (such as malasseziae) can show similar characteristics in the veterinary line, although this phenomenon might have a great impact in veterinary dermatology. It was reported in humans that in addition to *Candida* spp. also *M. pachydermatis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Pneumocystis* spp. and *Coccidioides immitis* might be able to produce fungal biofilms (Cannizzo *et al.*, 2007; Martinez *et al.*, 2010). Biofilms are extracellular matrices produced by microorganisms (primarily bacteria and fungi) and they help the pathogens attach to viable and non-viable surfaces (Van Minnebruggen *et al.*, 2010).

MBC is generally higher when examined in biofilm compared to the same bacteria in planktonic form. In a publication 14 *Pseudomonas aeruginosa* strains against tobramycin show 100 µg/ml MBC-B (biofilm) and 8 µg/ml MBC-P (planktonic) (Mah, 2014).

5. Materials and methods

5.1. Effect of the combination of marbofloxacin and gentamicin on the antimicrobial susceptibility of *Pseudomonas aeruginosa* strains

Bacteria involved in the study

A total of 68 *P. aeruginosa* strains isolated from dogs showing the clinical signs of otitis externa were used in this study. The origin of the strains were Hungarian and were collected from different territories. The strains were gained from Hungarian clinic *P. aeruginosa* strains were grown on MacConkey agar and were identified by Gram-staining, microscopic examination, growth in selective cefrimide agar (Scharlau Chemie, Sentmenat, Spain), colony morphology, pigment production and biochemical reactions. The strains were collected and stored at $-80\text{ }^{\circ}\text{C}$ in Mueller-Hinton broth (Biolab Zrt., Budapest, Hungary) supplemented with 20% sterile glycerol. Isolates were subcultured on 5% sheep blood agar plates directly before the investigation. A reference strain of *P. aeruginosa* (ATCC No. 27853) was used as quality control in consonance with Committee of Laboratory Standards Institute guideline (CLSI M07-A9. 2012).

***In vitro* susceptibility tests**

The broth microdilution method was performed in accordance with CLSI M7-A9. 2012 with a two-fold dilution in 96-well sterile microtiter plates. For the determination of MICs (minimum inhibitory concentrations) for the single antibiotics, final concentrations of marbofloxacin or gentamicin in the wells were set to 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.063 $\mu\text{g/ml}$ in Mueller-Hinton broth.

Bacteria grown for 24 hours in Mueller-Hinton broth were centrifuged at 3000 g for 10 minutes. The bacteria were washed and resuspended in physiological saline. The optical density of the suspension at 600 nm was set to 0.1 with the appropriate amount of physiological saline, that corresponded to 10^8 colony forming units (CFU) per ml. *P. aeruginosa* strains were diluted and distributed into the microtiter wells to achieve approximately a 10^5 CFU/ml bacterial density. The inoculated trays were incubated for a period of 24 h at $37\text{ }^{\circ}\text{C}$ and evaluated with the naked eye. Data were reported as

MIC ranges and MIC at which 90% of the strains were inhibited (MIC₉₀). Resistance threshold was determined according to CLSI and EUCAST (European Committee of Antimicrobial Susceptibility Testing) breakpoints. Isolates having MIC values of 4 µg/ml or higher for marbofloxacin, and 8 µg/ml or higher for gentamicin, were considered resistant. When evaluating the combination, the lower threshold was used.

Investigating antibiotic synergy

In order to describe interactions between marbofloxacin and gentamicin, the checkerboard microdilution method was utilized (Eliopoulos and Moellering, 1996; Bonapace *et al.*, 2000) for determining the lowest fractional inhibitory concentration (FIC) index (Hollander *et al.*, 1998). Similar to the single antibiotic susceptibility tests, *P. aeruginosa* strains were distributed in the microtiter wells to achieve a 10⁵ CFU/ml bacterial density. The inoculated trays were incubated for 24 h at 37 °C. The combined effects of the antibiotics were evaluated as synergy, addition, indifference, and antagonism. To evaluate these interactions, the values for marbofloxacin (FIC_M) and gentamicin (FIC_G) were determined at each dilution.

$$FIC_M = MIC_{M \text{ combination}} / MIC_{M \text{ alone}}$$

$$FIC_G = MIC_{G \text{ combination}} / MIC_{G \text{ alone}}$$

The FIC indices were calculated for each strain according to the used method (Eliopoulos, 1996) as $FIC_{\text{index}} = FIC_M + FIC_G$ and the results were interpreted as follows: synergy (<0.5), partial synergy/addition (0.5–1.0), indifference (1.0–4.0), and antagonism (>4.0). Mean FIC_{index} was calculated to analyse the interactions for all investigated bacteria (n=68) as

$$\text{Mean } FIC_{\text{index}} = \sum FIC_{\text{index}} / \text{Sum of strains}$$

In addition, data were also illustrated on isobolograms to confirm and analyse interactions between marbofloxacin and gentamicin. The isobologram, a graph of equally effective dose pairs, is a commonly used method to describe drug synergism. In these graphs, the MIC values of the single drugs and the different ratio combinations are plotted as axial points in a Cartesian plot. The straight line connecting $MIC_{\text{marbofloxacin}}$ and $MIC_{\text{gentamicin}}$ are those dose pairs that will produce an additive effect (Tallarida, 2001). Effective dose pairs found below the additive line are considered synergistic combinations, when confirmed with regression analysis.

5.2. Penetration of gentamicin and gentamicin supplemented with 1% DMSO across biological monolayer membranes *in vitro*

The IPEC-J2 cell line used in this study was derived from jejunal epithelia of a neonatal piglet and was a kind gift from Dr. Jody Gookin, North Carolina State University, USA. It is a non-transformed cell line that in some respects mimics *in vivo* conditions when cultured on membrane inserts. Cells form a differentiated monolayer and are attached to each other via tight junctions apically. The high transepithelial electrical resistance (TEER) of IPEC-J2 monolayers grown on Transwell polyester filters coated with rat tail collagen shows the functional integrity of a continuous, uninterrupted cell layer. Only monolayers with a TEER value of at least 8000 Ohm/cm² were used as a model system to study the cellular barrier permeability (Figure 2). Cells were maintained in complete medium containing 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) supplemented with 5% FBS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor and 1% penicillin-streptomycin (all from Fisher Scientific Inc., USA). Cell cultures were tested by PCR and were found to be free of mycoplasma contamination. IPEC-J2 cells were seeded at a density of $1.5 \cdot 10^5$ per well on six-well plates with Transwell polyester membrane inserts (pore size 0.4 µm; surface area 4.67 cm²; Sigma-Aldrich) coated with rat tail collagen (Sigma-Aldrich) in a 1.5 ml apical and 2.6 ml basolateral volume. Cells were allowed to adhere for 24 h before being washed and re-fed every other day until confluence. They were grown at 37 °C in a humidified atmosphere of 5% CO₂ for three weeks before the experiments. Three replicas were used for each treatment.

Prior to the treatments, IPEC-J2 cells were washed twice with plain medium (complete medium without FBS and antibiotics). Six-well inserts were treated apically with 1 mg/ml of gentamicin-sulphate (Sigma-Aldrich) and with gentamicin sulphate supplemented with 1% DMSO (Sigma-Aldrich) dissolved in plain medium. The basolateral compartments of the Transwell inserts were filled with plain medium. Treatment lasted for 1 and 6 h, and TEER was measured at different time points (before treatment and 6 h after treatment). One ml from each apical and basolateral medium was collected at different time points (0 h, 1 h, 6 h), diluted in PBS and the amount of gentamicin present in the apical and basolateral compartments was determined by HPLC.

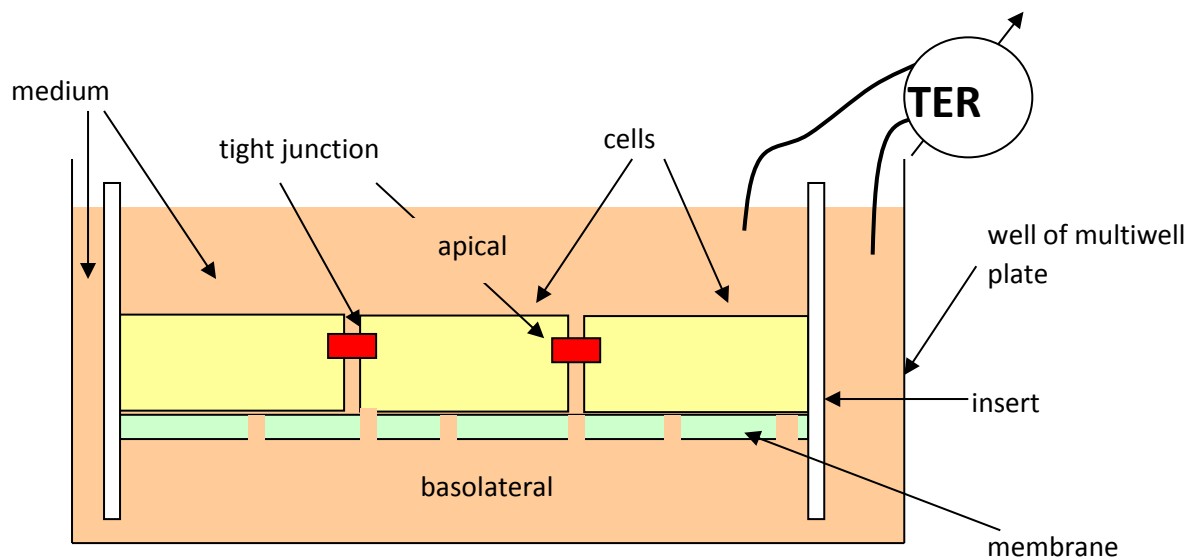


Figure 2. Experimental conditions. Polyester filters and cell culture monolayer fully isolating the apical and basolateral compartments in this system. Diffusion between these two compartments is only possible via transcellular or paracellular transport. Difference in the concentration of gentamicin between apical and basolateral compartments indicates transport mechanisms that take place through the cell monolayer.

For the determination of gentamicin concentration in the apical and basolateral compartments, a Merck LaChrome Elite HPLC system with a Spherisorb ODS-2 (5 µm pore size, 250 x 4.6 mm) column was used with fluorescent detection and an eluent consisting of 85% acetonitrile and 15% ultra-pure water. Temperature of the column and the autosampler was 25 °C and 20 °C, respectively. Excitation and emission wavelength of fluorescent detection were 260 nm and 315 nm, respectively. Flow rate was 1 ml/min, injection volume was 25 µl and the running time was 34 min. Preparation of standards (2, 1, 0.5, 0.2 and 0.1 µg/ml) was achieved with the dilution of 50 mg/ml gentamicin-sulphate (Sigma-Aldrich) standard 25 °C with ultra-pure water and a diluent consisting 50% acetonitrile (Prolabo, Thailand) and 50% borate buffer (Merck, Germany). The peak area and concentration showed a good linear relationship, while the concentration of gentamicin was between 0.1 µg/ml and 2 µg/ml, $r^2=0.9997$. DMEM/F12 medium, and DMEM/F12 supplemented with 1% DMSO were used as blank. Derivatization was achieved with 10 mM fluorenylmethyloxycarbonyl chloride (Merck) solution for 15 min. The reaction was stopped with 0.1 M glycine solution (Merck). The method was validated, limit of detection (LOD) was 0.19 ng/ml, limit of quantification (LOQ) was 0.63 ng/ml indicating a high sensitivity of the method.

For the statistical evaluation unpaired, two-sample Student's t-probe were used.

5.3. Effect of 8-day serial passage on the development of resistance against marbofloxacin–gentamicin (1:1) combination in selected *Pseudomonas aeruginosa* and *Staphylococcus pseudintermedius* strains

Pseudomonas aeruginosa

Bacterial strains and antimicrobial agents

Sixteen non-duplicate clinical isolates of *P. aeruginosa* were isolated from dogs and cats and were used in the present study. All animals subjected to sampling showed clinical signs of dermatitis or external otitis. Bacteria were isolated and cultured at DUO-BAKT Microbiological Laboratory, HUNGARY and the Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University. Bacteria were identified on the basis of their conventional biochemical

characteristics including colony characteristics, growth requirements, Gram staining, pigment production, and haemolytic properties. Only strains isolated from the skin were used in this study. The strains used in the present study were collected from canine ear canal from different Hungarian small animal clinics in 2009. Bacteria were kept frozen in Mueller-Hinton broth (Biolab Zrt., Budapest, Hungary) supplemented with 10% sterile glycerol at $-80\text{ }^{\circ}\text{C}$ during the entire *in vitro* preclinical study. Bacterial isolates were *resuscitated after* cryopreservation in Mueller-Hinton (MH) broth at an ambient temperature of $37\text{ }^{\circ}\text{C}$ for 24 hours before MIC determinations. Marbofloxacin and gentamicin were obtained from Sigma-Aldrich.

Preparation of bacterial suspensions

Bacteria were cultured in Mueller-Hinton (MH) broth for 24 hours and were centrifuged at 3000 g for 10 minutes. The supernatant was discarded and bacterial cells were washed with sterile physiological saline, centrifuged at 3000 g for 10 minutes and resuspended in physiological saline. Bacterial density was adjusted using an optical density (OD) of the suspension at 600 nm set to 0.1 ($\text{OD}_{600}=0.1$) using appropriate amount of physiological saline, which corresponded to 10^8 colony forming units (CFU)/ml and a standard of 0.5 on the McFarland scale. A suspension of 10^6 CFU/ml was prepared with a 100-fold dilution for the serial passage experiments. The colony count of the suspensions was tested by inoculation onto MH agar plates. Determination of MICs was in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) standards (CLSI M07-A9, 2012; Nascente *et al.*, 2009).

MIC determination

MICs of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination against the tested strains were initially determined. A twofold dilution was prepared from the stock solution in 96-well microplates. The final concentrations of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination (expressed in individual drug concentration) were set in each line of a new 96-well microplate to 128.0 $\mu\text{g/ml}$, 64.0 $\mu\text{g/ml}$, 32.0 $\mu\text{g/ml}$, 16.0 $\mu\text{g/ml}$, 8.0 $\mu\text{g/ml}$, 4.0 $\mu\text{g/ml}$, 2.0 $\mu\text{g/ml}$, 1.0 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$,

0.25 µg/ml by the use of working solutions. Bacteria were inoculated in 15 µl inoculum volume using a bacterial suspension (10⁶ CFU/ml). This resulted in a final bacterial density of 10⁵ CFU/ml. Positive control wells contained only Mueller-Hinton broth were inoculated with the given bacterial strain. Negative control wells contained Mueller-Hinton broth without inoculation. Plates were incubated for 24 hours at 37 °C.

Multistep studies

Following the determination of the MICs, bacteria were inoculated on the following day in a different microplate containing the same concentrations of marbofloxacin or the marbofloxacin–gentamicin (1:1) combination. For each strain, the highest concentration of the antibacterial where bacterial growth occurred was selected and from this well, bacteria were passaged to another plate with all of the concentrations present. Using this method, each bacterium was individually observed each day, and their colonies grown in the presence of the antibiotic (or antibiotic combination) were daily passaged to another. Every day the bacterium was inoculated onto a new plate from the well containing the highest concentration of the antibiotic. This method mimics the effect of subinhibitory concentrations present in biological systems and helps understand and interpret the risk posed by drug usage on the development of bacterial resistance.

Gene amplification and sequencing

For the detection of mutations in the genes of QRDRs as well as the efflux regulatory genes that are associated with fluoroquinolone or aminoglycoside resistance in *P. aeruginosa*, PCR amplification and sequencing were performed. One strain (strain No. 16) was chosen for the PCR studies, as it showed significant differences in MICs on the 8th day of passage (between MIC_{marbofloxacin} [32 µg/ml] and MIC_{marbofloxacin+gentamicin} [4 µg/ml]). Amplification and sequencing of *gyrA*, *gyrB*, *parC* and *parE* genes were performed as previously described (Akasaka *et al.*, 2001). Multiple efflux pump regulator genes (*nfxB*, *mexR*, *mexT*, *mexZ*) were amplified according to (Dougherty *et al.*, 2014). In addition, the examination of the intergenic region *mexOZ* is also included (Islam *et al.*, 2004).

CCCP assay

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma-Aldrich) is a proton conductor that causes uncoupling of the proton gradient that is maintained by the activity of electron carriers in the electron transport chain. CCCP acts as an efflux pump inhibitor in *P. aeruginosa* thereby increasing sensitivity to fluoroquinolones and other antimicrobials (Adabi *et al.*, 2015). In our study, CCCP was added at 25 µM to the microdilution assay in combination with the antibiotics studied.

Statistical analysis

Trends of MIC values were modelled with power functions of the days elapsed according to the following equation as follows: $MIC = \text{Base} \cdot \text{Days}^{\text{Exponent}}$. The parameters base and exponent were estimated for each treatment arm from the observed MIC values in the trial. Statistical analysis was fitted on the logarithmic scale where the trend appeared to be linear:

$\log(MIC) = \log(\text{Base}) + \text{Exponent} \cdot \log(\text{Days})$. The mixed model was applied where the random effects corresponded to *P. aeruginosa* strains (Brown and Prescott, 2006; Hothorn *et al.*, 2008).

Staphylococcus pseudintermedius

Thirty two (32) strains of *S. pseudintermedius* isolated from dogs and cats were investigated in this experiment. All of the animals subjected to sampling showed clinical signs of external otitis. Bacteria were isolated, cultured and supplied by DUO-BAKT Microbiological Laboratory. Bacteria were identified on the basis of colony characteristics, growth requirements, Gram-staining, haemolytic properties and biochemical reactions. The method and the preparation was the same as in case of *Pseudomonas aeruginosa* with the following difference: The final concentrations of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination (expressed in individual drug concentration) were set in each line of a new 96-well microplate to 16.0 µg/ml, 8.0 µg/ml, 4.0 µg/ml, 2.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 µg/ml and 0.03125 µg/ml with the exertion of working solutions.

5.4. Susceptibility of *Staphylococcus* spp., *Streptococcus canis* and *Pseudomonas aeruginosa* strains to marbofloxacin and susceptibility determination of *Malassezia pachydermatis* strains to ketoconazole

Microorganisms involved in the study in 2010

One hundred and twenty three (123) strains of staphylococci, thirty seven (37) strains of *S. canis* (Lancefield group G), fifty two (52) strains of *P. aeruginosa* and forty (40) strains of *M. pachydermatis* isolated from dogs and cats were investigated in this experiment. Bacteria were isolated, cultured and supplied by DUO-BAKT Microbiological Laboratory and the Szent István University, Faculty of Veterinary Science Department of Microbiology and Infectious Diseases. Some cat isolates were isolated from clinical samples in the Department of Pharmacology and Toxicology. Bacteria were identified on the basis of colony characteristics, growth requirements, Gram stain, pigment production, haemolytic properties and biochemical reactions. The bacteria and *Malassezia pachydermatis* involved in this study are shown in Table 1.

Microorganisms involved in this the between 2017–2018

Eighty nine (89) strains of *Staphylococcus pseudintermedius* and *S. aureus*, fifty five (55) strains of *P. aeruginosa* and eighty (80) strains of *M. pachydermatis* strains isolated from dogs were investigated in this experiment. Some strains of *Corynebacterium* spp., *Proteus mirabilis* and *streptococci*, *Candida* spp. and other fungi were also isolated in Hungary. Bacteria were sent from all over France and Hungary from different animal hospitals, and strains were collected, cultured and supplied by DUO-BAKT Microbiological Laboratory. Bacteria were identified on the basis of colony characteristics, growth requirements, Gram-stain, pigment production, haemolytic properties and biochemical reactions. The bacteria and *Malassezia pachydermatis* involved in this study are shown in Table 2.

Table 1. The bacteria involved in the study (2010)

Microorganism species	No. of isolates origin
	Hungarian
<i>Staphylococcus pseudintermedius</i>	129
<i>S. aureus</i>	6
<i>Streptococcus canis</i>	43
<i>Pseudomonas aeruginosa</i>	56
<i>Malassezia pachydermatis</i>	54

All strains were isolated from either the skin or the outer ear canal of dogs and cats showing the clinical signs of bacterial or fungal dermatitis or otitis externa.

Bacteria were kept on Mueller-Hinton bevelled agar (Biolab Zrt., Budapest, Hungary) broth at a temperature of 4 °C before the beginning of the study. Bacteria were propagated in Mueller-Hinton (MH) broth at an ambient temperature of 37 °C for 24 hours before the MIC-determination. Streptococci were kept on agar containing 5% defibrinated sheep blood at a temperature of 4 °C before the beginning of the study. Streptococci were propagated in Brain Heart Infusion (BHI) (Biolab Zrt., Budapest, Hungary) broth at temperature of 37 °C for 24 hours before the MIC-determination.

M. pachydermatis strains were kept on „Sabouraud dextrose with chloramphenicol” (Biolab Zrt., Budapest, Hungary) agar broth at a temperature of 4 °C before the beginning of the study. Fungi were propagated in Sabouraud dextrose broth with chloramphenicol at temperature of 37 °C for 72 hours before the MIC-determination.

Table 2. The bacteria involved in the study (2017–2018)

Microorganism species	No. of isolates origin	
	French	Hungarian
<i>Staphylococcus pseudintermedius</i> and <i>S. aureus</i>	41	48
<i>Streptococcus canis</i>	0	22
<i>Pseudomonas aeruginosa</i>	23	32
<i>Proteus mirabilis</i>	0	1
<i>Escherichia coli</i>	0	28
<i>Corynebacterium</i> spp.	0	1
<i>Pasteurella multocida</i>	0	1
<i>Malassezia pachydermatis</i>	41	39
<i>Candida</i> and other yeasts	0	8

Preparation of bacterial suspensions

Bacteria multiplied in Mueller-Hinton (MH) broth for 24 hours were centrifuged at 3000 g for 10 minutes. The bacteria were washed with sterile physiological saline, centrifuged at 3000 g for 10 minutes and resuspended in physiological saline. The optical density at 600 nm of the suspension were set to 0.1 ($OD_{600}=0.1$) with the appropriate amount of physiological saline, that corresponds with 10^8 colony forming unit (CFU)/ml bacterial density and a standard of 0.5 on the McFarland scale. The bacterial density – optical density calibration curve is shown in Figure 3 (showing the optical densities of a *S. pseudintermedius* strain).

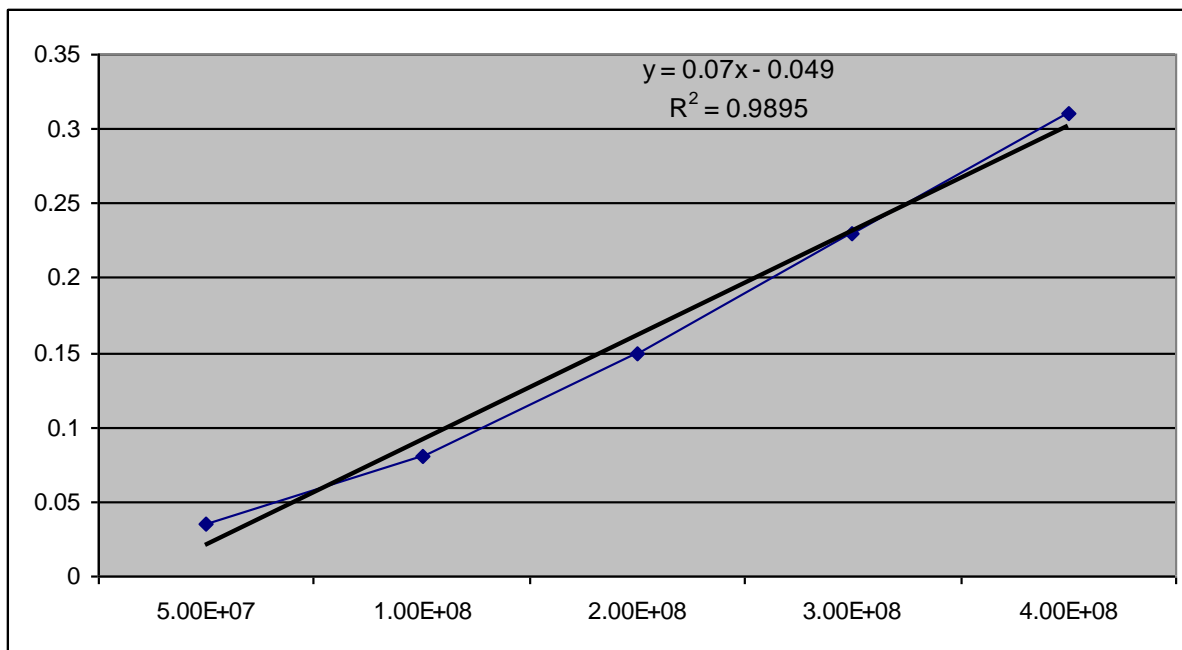


Figure 3. Calibration curve for determination of bacterial concentration from optical density at 600 nm (this particular is derived from *S. pseudintermedius* strain No. 1). Y axis shows optical density, X axis shows bacterial density.

A suspension of 10^6 CFU/ml was prepared with a 100-fold dilution. The germ-count of the suspensions was tested with inoculation to agar plates and counting the number of CFU.

Preparation of fungal suspensions

M. pachydermatis strains multiplied in Sabouraud broth (Biolab Zrt., Budapest, Hungary) for 48 hours were centrifuged at 3000 g for 10 minutes. The fungi were washed with sterile physiological saline, centrifuged at 3000 g for 10 minutes and resuspended in physiological saline. The optical density at 600 nm of the suspension was set to 0.1 ($OD_{600}=0.25$) with the appropriate amount of physiological saline, that corresponds to 10^7 colony forming unit (CFU)/ml fungal density and a McFarland 1 standard.

A suspension of approximately 10^6 CFU/ml was prepared with a 10-fold dilution. The germ-count of the suspensions was tested with inoculation to Sabouraud agar plates and counting the number of CFU.

Preparation of stock solutions

Marbofloxacin stock solution at concentration of 1600 µg/ml was prepared with dissolving 16.1 mg of marbofloxacin (Menovo Pharmaceutical Co. Ltd., Zhejiang, China; Batch No.: MBX-9-090902; Manufacture date: 08.09.2009.; active substance content 99.3%) in 10 ml of sterile distilled water. Marbofloxacin stock solution was sterilized with filtration through a 0.22 µm membrane filter and stored at –80 °C.

Ketoconazole stock solution at concentration of 1600 µg/ml concentration was prepared with dissolving 16.1 mg of ketoconazole (Nanjing Baijingyu Pharmaceutical Co. Ltd., Nanjing, China; Batch No.: KE09003; Manufacture date: 24.10.2009.; assay on dried basis 98.9%) in 10 ml of sterile dimethyl sulphoxide (DMSO).

Determination of MIC_{marbofloxacin}-values with broth microdilution

For the determination of MICs for marbofloxacin in staphylococci, a two-fold dilution was prepared from the stock solution in 96-well microplates. In the first line the stock solution was diluted five-fold to obtain 320 µg/ml concentration of marbofloxacin. This was performed by diluting 40 µl of the marbofloxacin stock solution with 160 µl of Mueller-Hinton broth. Two-fold dilution with MH-broth was prepared in each line to achieve working solutions with 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 µg/ml concentrations of marbofloxacin in accordance with Figure 4.

The final concentrations of marbofloxacin were set in each line of a new 96-well microplate to 32.0 µg/ml, 16.0 µg/ml, 8.0 µg/ml, 4.0 µg/ml, 2.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 µg/ml with the exertion of the working solutions. This was achieved by 10-fold dilution of the working solutions with pipetting 15 µl to 120 µl of Mueller-Hinton broth. The final step was the inoculation of 15 µl of the bacterium-suspension (10^6 CFU/ml) resulted in the final bacterial density of 10^5 CFU/ml. Final concentrations of marbofloxacin in the microplates are indicated in Figure 5. Positive control wells contained only Mueller-Hinton broth and were inoculated with the certain bacterial strain. Negative control wells contained Mueller-Hinton broth without inoculation.

320	160	80	40	20	10	5	2.5	1.25	0.625			1
320	160	80	40	20	10	5	2.5	1.25	0.625			2
320	160	80	40	20	10	5	2.5	1.25	0.625			3
320	160	80	40	20	10	5	2.5	1.25	0.625			4
320	160	80	40	20	10	5	2.5	1.25	0.625			5
320	160	80	40	20	10	5	2.5	1.25	0.625			6
320	160	80	40	20	10	5	2.5	1.25	0.625			7
320	160	80	40	20	10	5	2.5	1.25	0.625			8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 4. Working solutions for marbofloxacin in 96-well microplates (concentration values in $\mu\text{g/ml}$, each well corresponds to 200 μl volume) for staphylococci

32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	1
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	2
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	3
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	4
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	5
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	6
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	7
32	16	8	4	2	1	0.5	0.25	0.125	0.0625	Pos. contr.	Neg. contr.	8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 5. Final concentrations of marbofloxacin in 96-well microplates with the application of positive and negative controls (concentration values in $\mu\text{g/ml}$, each well corresponds to 150 μl volume) - *staphylococci*

For the determination of MIC for marbofloxacin in less susceptible bacteria (*S. canis* and *P. aeruginosa*) a different dilution was used. In the first line the stock solution was diluted to obtain 640 µg/ml concentration of marbofloxacin. This was performed by diluting 80 µl of the marbofloxacin stock solution with 120 µl of Mueller-Hinton broth in the case of *P. aeruginosa* and BHI medium in the case of *S. canis*. Two-fold dilution with the broths was prepared in each line to achieve working solutions with 640, 320, 160, 80, 40, 20, 10, 5, 2.5 and 1.25 µg/ml concentrations of marbofloxacin in accordance with Figure 6.

640	320	160	80	40	20	10	5	2.5	1.25			1
640	320	160	80	40	20	10	5	2.5	1.25			2
640	320	160	80	40	20	10	5	2.5	1.25			3
640	320	160	80	40	20	10	5	2.5	1.25			4
640	320	160	80	40	20	10	5	2.5	1.25			5
640	320	160	80	40	20	10	5	2.5	1.25			6
640	320	160	80	40	20	10	5	2.5	1.25			7
640	320	160	80	40	20	10	5	2.5	1.25			8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 6. Working solutions for marbofloxacin in 96-well microplates (concentration values in µg/ml, each well corresponds to 200 µl volume) for *S. canis* and *P. aeruginosa*

The final concentrations of marbofloxacin were set in each line of a new 96-well microplate to 64.0 µg/ml, 32.0 µg/ml, 16.0 µg/ml, 8.0 µg/ml, 4.0 µg/ml, 2.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml with the exertion of the working solutions. This was achieved by 10-fold dilution of the working solutions with pipetting 15 µl to 120 µl of MH or BHI medium. The final step was the inoculation of 15 µl of the

bacterium-suspension (10^6 CFU/ml) resulted in the final bacterial density of 10^5 CFU/ml. Final concentrations of marbofloxacin in the microplates are indicated in Figure 7. Positive control wells contained only Mueller-Hinton or BHI medium and were inoculated with the certain bacterial strain. Negative control wells contained Mueller-Hinton or BHI medium without inoculation.

64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	1
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	2
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	3
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	4
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	5
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	6
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	7
64	32	16	8	4	2	1	0.5	0.25	0.125	Pos. contr.	Neg. contr.	8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 7. Final concentrations of marbofloxacin in 96-well microplates with the application of positive and negative controls (concentration values in $\mu\text{g/ml}$, each well corresponds to $150\ \mu\text{l}$ volume) – *S. canis*, *P. aeruginosa*

After the inoculation of the bacteria the microplates were placed in thermostat with a temperature of $37\ ^\circ\text{C}$ for 16 hours. After the incubation period the bacterial growth was examined with the unaided eye. Statements for each well:

+++ = pronounced growth of the bacteria

++ = moderate growth of the bacteria

+ = low growth potency of the bacteria

\pm = „faint haze”, no evidence for bacterial growth

– = no growth

In the evaluation procedure - and \pm wells were considered negative, +, ++ and +++ were considered positive.

Being aware of the bacterial growth intensities experienced at different drug concentrations the MIC-values were determined for marbofloxacin of each strain.

Determination of MIC_{ketoconazole}-values with broth microdilution

For the determination of MIC for ketoconazole in malasseziae, a two-fold dilution was prepared from the stock solution in 96-well microplates. In the first line the stock solution was diluted ten-fold to obtain 160 $\mu\text{g/ml}$ concentration of ketoconazole. This was performed by diluting 20 μl of the ketoconazole stock solution with 180 μl of Sabouraud broth. Two-fold dilution with Sabouraud broth was prepared in each line to achieve working solutions with 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 $\mu\text{g/ml}$ concentrations of ketoconazole in accordance with Figure 8.

160	80	40	20	10	5	2.5	1.25	0.625	0.3125			1
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			2
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			3
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			4
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			5
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			6
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			7
160	80	40	20	10	5	2.5	1.25	0.625	0.3125			8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 8. Working solutions for ketoconazole in 96-well microplates (concentration values in $\mu\text{g/ml}$, each well corresponds to 200 μl volume)

The final concentrations of ketoconazole were set in each line of a new 96-well microplate to 16.0 µg/ml, 8.0 µg/ml, 4.0 µg/ml, 2.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 µg/ml and 0.03125 µg/ml with the exertion of the working solutions. This was achieved by 10-fold dilution of the working solutions with pipetting 15 µl to 120 µl of Sabouraud broth. The final step was the inoculation of 15 µl of the yeast suspension (10^6 CFU/ml) resulted in the final density of 10^5 CFU/ml. Final concentrations of ketoconazole in the microplates are indicated in Figure 9. Positive control wells contained only Sabouraud broth and were inoculated with the certain strain of yeast. Negative control wells contained Sabouraud broth without inoculation.

16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	1
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	2
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	3
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	4
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	5
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	6
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	7
16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	Pos. contr.	Neg. contr.	8
1	2	3	4	5	6	7	8	9	10	11	12	

Figure 9. Final concentrations of ketoconazole in 96-well microplates with the application of positive and negative controls (concentration values in µg/ml, each well corresponds to 150 µl volume)

After the inoculation of the yeasts the microplates were placed in thermostat with a temperature of 37 °C for 72 hours. After the incubation period the growth was examined with the unaided eye described under 3.3.2.

5.5. Activity of ketoconazole against biofilm producing and planktonic *Malassezia pachydermatis* strains

Yeasts involved in this study

Eight strains of *Malassezia pachydermatis* strains were involved in this experiment. All of the strains were isolated from dogs suffering from clinical signs of malassezia infections of the skin or the ear. Strains were acquired from DUO-BAKT Microbiological Laboratory in 2009–2010 (Table 3).

Table 3. *Malassezia pachydermatis* strains involved in the study.

No.	Yeast	Host	Country	Year of isolation
3	<i>M. pachydermatis</i>	dog	Hungary	2009
9	<i>M. pachydermatis</i>	dog	Hungary	2009
10	<i>M. pachydermatis</i>	dog	Hungary	2009
13	<i>M. pachydermatis</i>	dog	Hungary	2009
16	<i>M. pachydermatis</i>	dog	Hungary	2009
18	<i>M. pachydermatis</i>	dog	Hungary	2009
20	<i>M. pachydermatis</i>	dog	Hungary	2009
43	<i>M. pachydermatis</i>	dog	Hungary	2010

M. pachydermatis strains were kept on „Sabouraud dextrose with chloramphenicol” (Biolab Zrt., Budapest, Hungary) broth at a temperature of 4 °C before the beginning of the study. Fungi were propagated in Sabouraud dextrose broth with chloramphenicol at temperature of 37 °C for 72 hours before the MIC-determination.

Preparation of fungal suspensions

M. pachydermatis strains multiplied in Sabouraud broth (Biolab Zrt., Budapest, Hungary) for 48 hours were centrifuged at 3000 g for 10 minutes. The fungi were washed with sterile physiological saline, centrifuged at 3000 g for 10 minutes and resuspended in physiological saline. The optical density at 600 nm of the suspension was set to 0.1 ($OD_{600}=0.25$) with the appropriate amount of physiological saline, that corresponds to 10^7 colony forming unit (CFU/ml) fungal density and a McFarland 1 standard.

A suspension of approximately 10^6 CFU/ml was prepared with a 10-fold dilution. The germ-count of the suspensions was tested with inoculation to Sabouraud agar plates and counting the number of CFU.

Biofilm production

Fungal biofilms are frequently produced on the surface of intravenous, urinary and other types of catheters made from polyurethane or polystyrol. In this study a sterile, disposable Foley catheter was used. Standardized segments of 10 mm tubes were prepared from the catheter and sliced into half under laminar flow. The surface of these catheters were scraped with sterile, gamma sterilised scalpel blades to enhance attachment of the yeasts. The 10 mm catheter segments were placed in 24-well sterile Corning Costar microplates in lines 1 and 3. Lines 2 and 4 served as planktonic controls for each strain without any catheter segment.

Biofilm production: 25 μ l of a standardized (10^6 CFU/ml) yeast suspension was dropped on the surface of the catheter segments and were put in a thermostat at 37 °C for 1 hour (attachment phase) to help adhering of the yeasts to the catheter surface. After 1 hour these segments (along with the yeasts on the surface) were submerged in 1 ml of Sabouraud broth including different concentrations of ketoconazole. Fungal suspensions were placed in a thermostat at 37 °C for 72 hours.

Planktonic forms of yeasts: in these cases no attachment phase was utilised, the fungi were directly inoculated into Sabouraud broth containing different concentrations of ketoconazole. Thus, the organisms remained in planktonic forms without any attachment. Fungal suspensions were placed in a thermostat at 37 °C for 72 hours.

Determination of MIC-values of ketoconazole with broth microdilution in *M. pachydermatis* strains

Ketoconazole stock solution at concentration of 1600 µg/ml concentration was prepared with dissolving 16.1 mg of ketoconazole (Nanjing Baijingyu Pharmaceutical Co. Ltd., Nanjing, China; Batch No.: KE09003; Manufacture date: 24.10.2009.; assay on dried basis 98.9%) in 10 ml of sterile dimethyl sulphoxide (DMSO).

Determination of MIC_{ketoconazole}-values with the unaided eye in planktonic and biofilm producing systems

For the determination of MIC for ketoconazole in malasseziae, a five-fold dilution was prepared from the stock solution in 24-well microplates.

The different concentrations of ketoconazole solutions were prepared in 10 ml Wassermann tubes and 1–1 ml was pipetted to the microplate. Concentrations were set to 25, 5, 1, 0.2, 0.04 and 0.008 µg/ml.

The 25 µg /ml solution was prepared with a 64-times dilution of the ketoconazole stock solution (1600 µg /ml). This was achieved by pipetting 100 µl of the stock solution into 6300 µl of Sabouraud broth. The other dilutions were achieved by diluting 1 ml of the previous member of the dilution series with 4 ml of Sabouraud broth.

Alternated inoculations were made in each line: in lines 1 and 3 two different strains were inoculated on biofilms. In lines 2 and 4 the same two strains were inoculated without biofilms. Thus, 1–2, and 3–4 lines contained the same yeast strains, respectively. This arrangement facilitated comparison between biofilm forming and planktonic yeast strains. The arrangement of 24-well plates are shown in Figure 10. and Figure 11.

1	Strain No. X in biofilm	0.008	0.04	0.2	1	5	25
2	Strain No. X without biofilm	0.008	0.04	0.2	1	5	25
3	Strain No. Y. in biofilm	0.008	0.04	0.2	1	5	25
4	Strain No. Y without biofilm	0.008	0.04	0.2	1	5	25

Figure 10. Layout of a 24-well plate. Numbers represent ketoconazole concentrations in $\mu\text{g/ml}$. No. X and No. Y are two different strains of *M. pachydermatis*. Odd numbered lines include biofilm producing, even numbered lines include planktonic forms of the same strains.

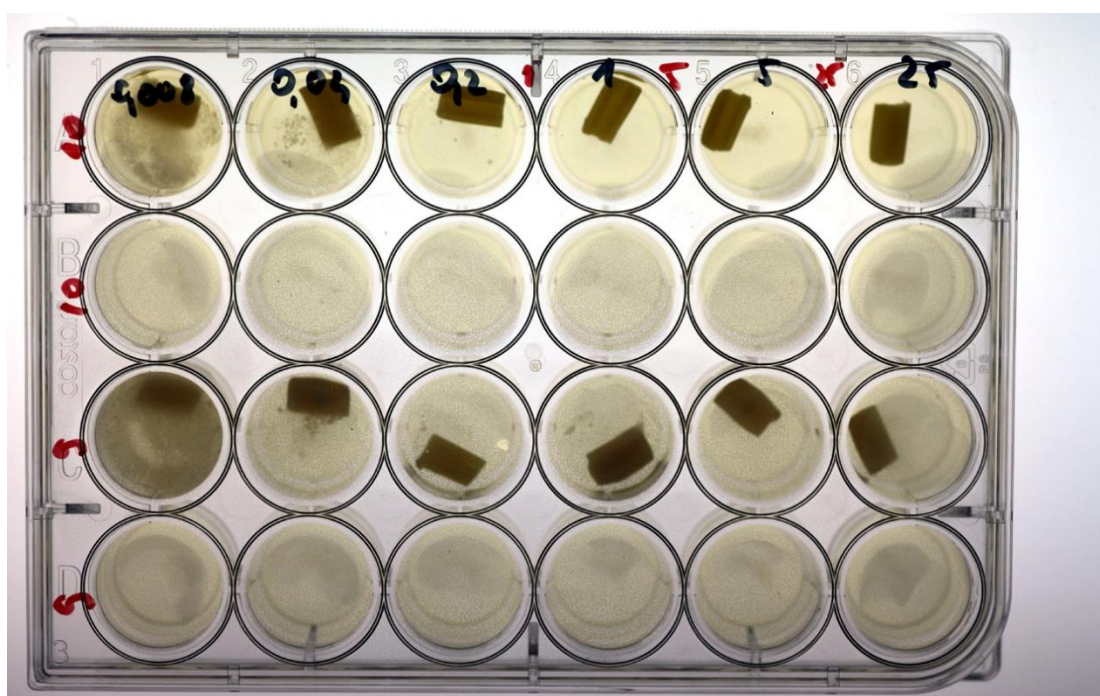


Figure 11. Growth of *M. pachydermatis* strains No. 9 and 10 in the presence and without the presence of biofilms. Line 1 and 3 contain biofilm producing forms, line 2 and 4 contain the planktonic forms. Ketoconazole concentrations range from 0.008 to 25 $\mu\text{g/ml}$

Positive control wells contained only Sabouraud broth and were inoculated with the certain strain of yeast. Negative control wells contained Sabouraud broth without inoculation (not included in Figure 10 and 11).

After the inoculation of the yeasts the microplates were placed in thermostat with a temperature of 37 °C for 72 hours. After the incubation period the growth was examined with the unaided eye. Each well and the fungal suspension was described with one of the following statements:

- +++ = pronounced growth of the yeast
- ++ = moderate growth of the yeast
- + = low growth potency of the yeast
- ± = „faint haze”, no evidence for yeast growth
- = no growth

In the evaluation procedure – and ± tubes were considered negative, +, ++ and +++ were considered positive.

Being aware of the fungal growth intensities experienced at different drug concentrations the MIC-values were determined for ketoconazole of each strain. MIC (minimum inhibitory concentration) is the smallest concentration of the antifungal agent, where no growth of the certain strain could be observed.

Determination of MIC_{ketoconazole}-values with the Celltiter96® Aqueous Proliferation Assay (MTS) in planktonic and biofilm producing systems

For a much more precise determination of MIC for ketoconazole in malasseziae, and to determine MFC (minimum fungicidal concentration) of ketoconazole a five-fold dilution was prepared from the stock solution in 24-well microplates as described under 3.4.3. Concentrations were set similarly to 25, 5, 1, 0.2, 0.04 and 0.008 µg/ml.

The initial steps were prepared in a similar manner as in case of the unaided eye examinations:

A 25 µg/ml solution was prepared with a 64-times dilution of the ketoconazole stock solution (1600 µg/ml). This was achieved by pipetting 100 µl of the stock solution into 6300 µl of Sabouraud broth. The other dilutions were achieved by diluting 1 ml of the previous member of the dilution series with 4 ml of Sabouraud broth. Alternated

inoculations were made in each line: in lines 1 and 3 two different strains were inoculated on biofilms. In lines 2 and 4 the same two strains were inoculated without biofilms. Thus, 1–2, and 3–4 lines contained the same yeast strains, respectively. This arrangement facilitated comparisons between biofilm forming and planktonic yeast strains. The arrangement of 24-well plates are shown in Figure 10.

After the inoculation of the yeasts the microplates were placed in thermostat with a temperature of 37 °C for 72 hours. After the incubation period the growth was examined with the *Celltiter96® Aqueous Proliferation Assay* containing the methyl-tetrazolium reagent. This assay is appropriate to determine viable cell count in different biosystems.

The *Celltiter96® One Solution Cell Proliferation Assay* is a colorimetric method to determine the number of viable cells in proliferation or cytotoxicity assays. The *Celltiter96® Aqueous One Solution Reagent* contains a novel tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-4-sulfophenyl-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution.”

The MTS tetrazolium compound (Owen’s reagent) is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding a small amount of the reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

During the measurements in ELISA reader (at 490 nm) blank samples were also utilized. These wells contained the Sabouraud broth with the *Celltiter96® Aqueous reagent*. The ELISA reader software automatically corrected the real samples with the absorption values of the blank.

As a consequence to these attributes, the MIC levels could be determined much more precisely and the investigator can get an accurate picture about the amount of living cells, thus the MFC (minimum fungicidal concentration).

6. Results

6.1. Effect of the combination of marbofloxacin and gentamicin on the antimicrobial susceptibility of *Pseudomonas aeruginosa* strains

The sensitivity of the investigated *P. aeruginosa* strains to the two investigated antimicrobials applied as a single treatment varied within broad limits (Table 4). The MICs for marbofloxacin and gentamicin ranged from 0.25–64 µg/ml and 0.25–32 µg/ml, respectively. Three strains were resistant to both antimicrobials alone, but two of these isolates were susceptible to the combination. The reference strain (ATCC No. 27853) showed MICs of 2 µg/ml for marbofloxacin as well as gentamicin. With respect to CLSI and EUCAST breakpoints, the ratio of resistant strains was 36.8% and 10.3% for marbofloxacin and gentamicin, respectively.

Table 4. Distribution of minimum inhibitory concentrations (MICs), MIC₉₀ values and percentage of resistant strains in case of marbofloxacin, gentamicin and their 1:1 combination against *P. aeruginosa* isolated from dogs (n=68).

	No. of isolates with MIC (µg/ml)												MIC ₉₀ (µg/ml)	% of resistant strains*	Mean FIC index
	128	64	32	16	8	4	2	1	0.5	0.25	0.13	0.06			
Marbofloxacin	0	4	0	7	8	6	23	11	8	1	0	0	16	36.8	
Gentamicin	0	0	4	0	3	17	27	14	2	1	0	0	8	10.3	
Marbofloxacin– gentamicin (1:1) combination	0	0	0	0	1	0	4	10	33	17	1	2	1	1.5	0.546

Four gentamicin resistant strains were sensitive and 3 strains were resistant to marbofloxacin, of which 2 proved to be susceptible to the combination (Table 5).

Regarding the combination, the FIC indices ranged from 0.0945 (pronounced synergy) to 1.0625 (indifference). Additive and indifferent interactions were observed in 31 and 4 strains, respectively. Synergy between marbofloxacin and gentamicin was found in 33 isolates, while no antagonistic effect was observed in any of the strains. Taking into account the FIC indices acquired with the checkerboard method and isobologram analysis, the 1:1 ratio of the antimicrobials proved to be the most effective.

Table 5. Minimum inhibitory concentrations of gentamicin resistant strains to marbofloxacin, gentamicin and the 1:1 ratio combination. Grey cells represent resistance according to CLSI breakpoints. Synergism between the active ingredients is described as partial ($1 > \text{FIC} > 0.5$) or full ($\text{FIC} < 0.5$) synergy

Strain No.	MIC _{marbofloxacin}	MIC _{gentamicin}	MIC _{combination}	FIC	Synergy
1	2	8	1	0.625	Partial
2	0.5	8	0.5	1.0625	None
3	1	32	0.5	0.515625	Partial
4	2	8	0.5	0.3125	Full
5	4	32	0.5	0.140625	Full
6	16	32	8	0.75	Partial
7	4	32	2	0.5625	Partial

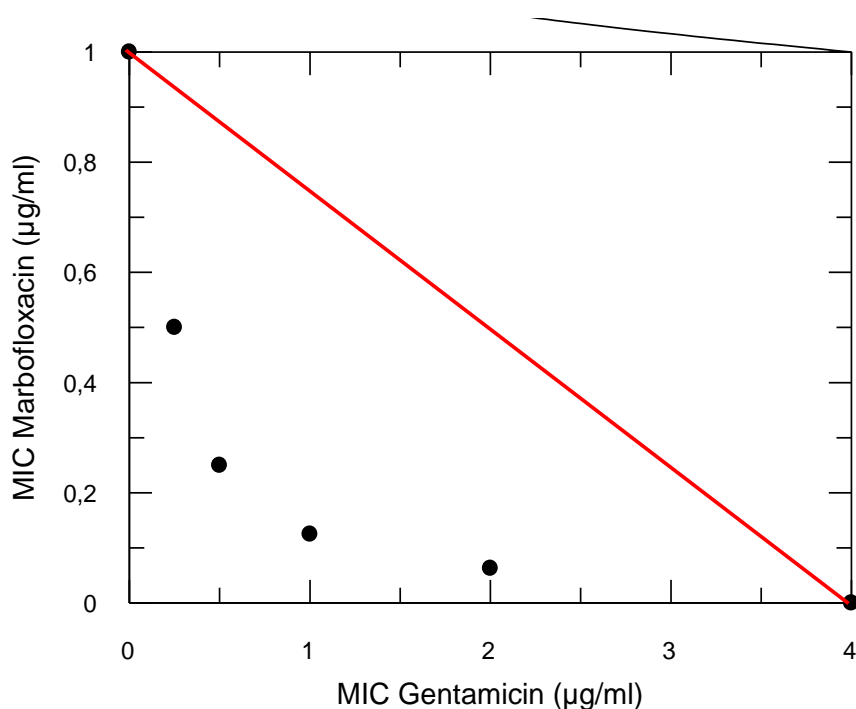


Figure 12. Sample isobologram of marbofloxacin and gentamicin in one *P. aeruginosa* strain. Convex curves (points below the additive line) indicate synergy, concave curves (points above the additive line) show antagonistic effect between two antimicrobials in a certain strain

The feature of this fixed ratio combination is its much better activity against *P. aeruginosa* with a MIC range of 0.031–8 µg/ml and a MIC₉₀ of 1 µg/ml compared to the 16 and 8 µg/ml values for marbofloxacin and gentamicin alone, respectively. Only 1 strain (1.5%) was resistant to the combination. The mean FIC index for the investigated *P. aeruginosa* strains was 0.546, which represents a partial synergistic/additive effect close to the full synergy threshold. The isobolograms acquired were convex without any exception, showing no antagonistic effect between the two antimicrobials in any of the isolates (Figure 12).

6.2. Penetration of gentamicin and gentamicin supplemented with 1% DMSO across biological monolayer membranes *in vitro*

Previous studies have shown that IPEC-J2 cells are able to differentiate with a parallel expression of tight junction proteins and increasing TEER values (Schierack *et al.*, 2006) forming a cell monolayer that acts as a barrier against polar substances of high molecular weight. Claudins and E-cadherin have been considered as key integral protein regulators responsible for maintenance of electrical resistance and paracellular integrity (Oliveira *et al.*, 2007). Previous findings indicate that certain solvents, like the surfactant labrasol can disrupt tight junction integrity, and thus enhance the penetration of gentamicin across membranes (Hu *et al.*, 2001). This phenomenon was not observed when gentamicin was supplemented with other solvent, 1% DMSO.

To assess the effect of the substances on the integrity of the cell monolayer, TEER was determined before and after the treatment. TEER was measured and was consistently above 8000 Ohm/cm² before treatment and 6 h after treatment. Figure 13 shows the observed TEER values of gentamicin and gentamicin + 1% DMSO treated IPEC-J2 cell cultures before and after 6 hours of treatment.

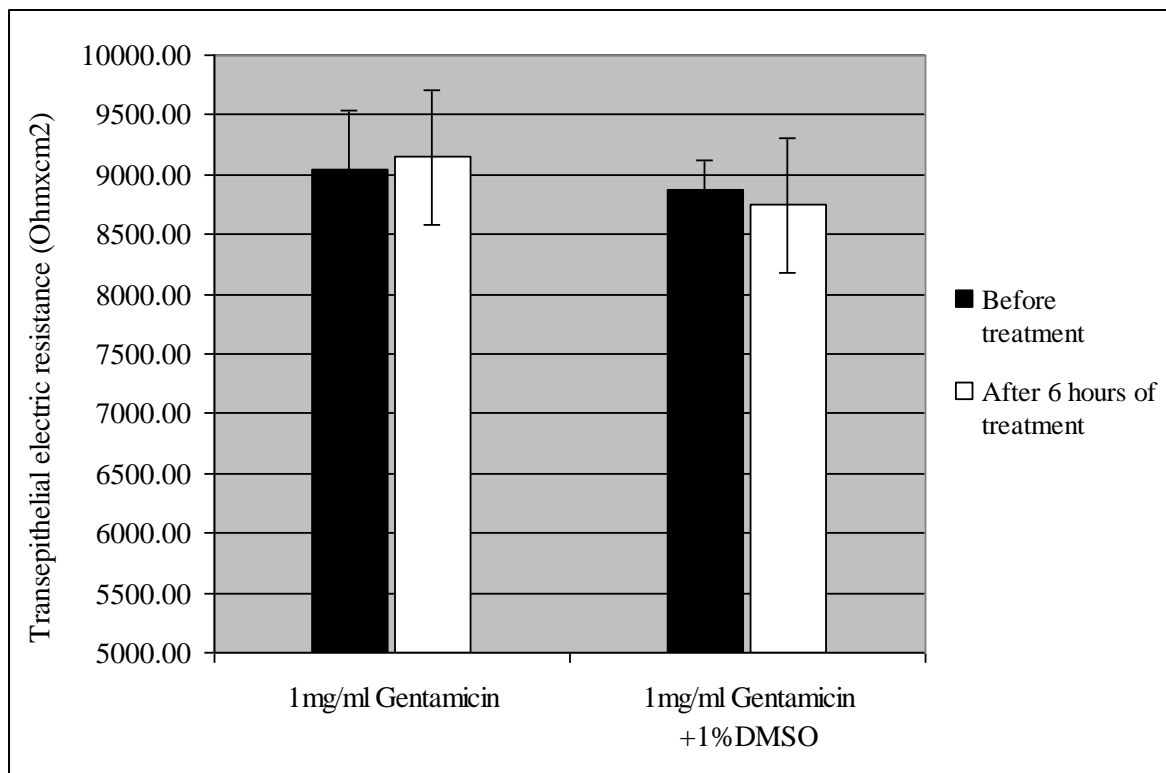


Figure 13. Transepithelial electric resistance (TEER) values before treatment and after 6 hours of treatment with 1 mg/ml gentamicin and 1 mg/ml gentamicin supplemented with 1% DMSO (mean±SD).

There was no significant difference ($p>0.05$) between TEERs before the treatment and 6 h after treatment with gentamicin or gentamicin in 1% DMSO, thus it is assumed that cell integrity was damaged neither by 1 mg/ml gentamicin nor by gentamicin supplemented with 1% DMSO. TEER values show that neither gentamicin nor gentamicin supplemented with 1% DMSO alters tight junction formation and paracellular integrity. It was previously described (Konari *et al.*, 1995) that DMSO can even induce the expression of tight junction proteins and thus increase TEER values. At the low concentration of DMSO used in this study, this phenomenon was not observed.

Table 6. shows the transport of gentamicin in the presence and absence of 1% DMSO in insert without cells and across the IPEC-J2 cell monolayer. In wells without cells, almost total equilibrium in gentamicin concentration was achieved after 6 h between apical and basolateral compartments. Addition of 1% DMSO did not alter gentamicin transport significantly ($p>0.05$).

Table 6. Concentrations of gentamicin (%) in apical and basolateral compartments in wells containing IPEC-J2 cell monolayer and in control wells containing no cells. 100% represents initial apical gentamicin concentration. Significant differences (* $p < 0.05$) in gentamicin permeation were found between initial value at $t = 0$ h and $t = 1$ h or $t = 6$ h when insert was not covered with cell layer. Data are shown as means \pm S. E. Ms ($n = 6$)

Treatment	Time (h)	Insert without cells		Cell monolayer	
		Apical compartment (%)	Basolateral compartment (%)	Apical compartment (%)	Basolateral compartment (%)
Gentamicin	0	99.85 \pm 0.24	0.00 \pm 0.29	98.36 \pm 0.90	0.15 \pm 0.12
	1	72.38 \pm 1.07*	26.47 \pm 1.52*	99.13 \pm 0.85	0.05 \pm 0.23
	6	52.13 \pm 0.53*	46.81 \pm 0.32*	99.94 \pm 0.01	1.12 \pm 0.81
Gentamicin in 1% DMSO	0	99.65 \pm 0.62	0.15 \pm 0.29	99.54 \pm 0.21	0.41 \pm 0.08
	1	72.67 \pm 1.15*	27.12 \pm 1.29*	98.56 \pm 0.91	0.11 \pm 0.05
	6	54.45 \pm 1.23*	44.98 \pm 1.31*	99.93 \pm 0.02	0.05 \pm 0.01

The membrane insert served as a time-dependent barrier as it delayed the equilibration of gentamicin concentrations between the two compartments. In wells with cell monolayer, no gentamicin was detected in the basolateral compartment either in wells treated with gentamicin, or in wells treated with gentamicin + 1% DMSO, 1 h after treatment, and the total gentamicin content was detected in apical region (Figure 14).

Six h after treatment, a very low amount of gentamicin was measured in the basolateral compartment. No significant difference ($p > 0.05$) was observed in gentamicin levels in wells treated with gentamicin alone, or in combination with 1% DMSO. Thus, gentamicin shows poor transcellular and paracellular permeation across a cell monolayer *in vitro*. DMSO added in 1% concentration did not enhance the permeation of the aminoglycoside through cell monolayers. Therefore, it can be assumed that 1% DMSO does not significantly alter gentamicin penetration across monolayer biological membranes *in vitro*. According to TER and HPLC measurements it is proved that DMSO in 1% concentration does not alter paracellular integrity and thus gentamicin permeation in IPEC-J2 cells.

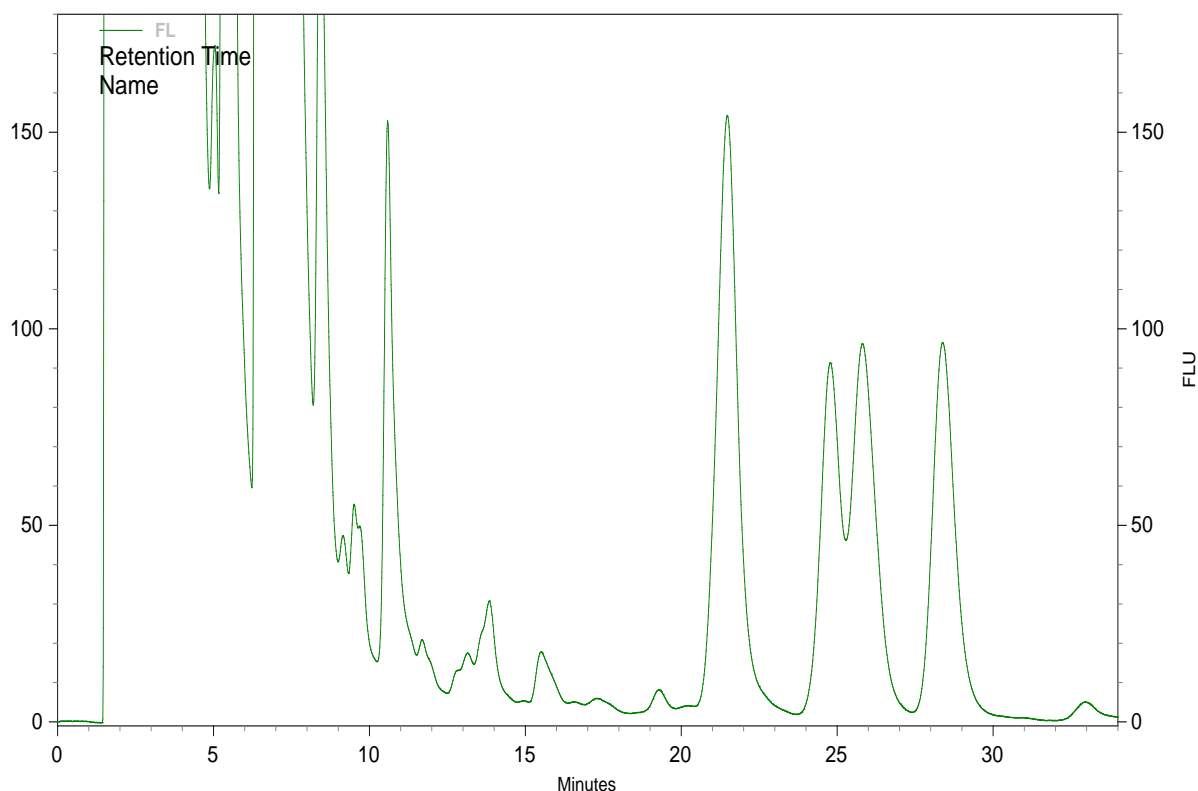


Figure 14. Representative chromatogram of gentamicin complex collected from apical compartment of IPEC-J2 cell monolayer. The order of elution with mobile phase (85% ACN, 15% UPW) was gentamicin C1 (tret=21.89 min), gentamicin C1a (tret=24.7 min), gentamicin C2a (tret=25.67 min) and gentamicin C2 (tret=28.42 min). The fluorescence detection wavelengths were 260 nm (excitation λ) and 315 nm (emission λ). The flow rate was adjusted to 1 ml/min

6.3. Effect of 8-day serial passage on the development of resistance against marbofloxacin–gentamicin (1:1) combination in selected *Pseudomonas aeruginosa* and *Staphylococcus pseudintermedius* strains

Pseudomonas aeruginosa

The minimum inhibitory concentrations (MICs) of marbofloxacin and the marbofloxacin- gentamicin (1:1) combination in sixteen *P. aeruginosa* strains during the 7 passages are shown in Table 7.

Table 7. Minimum inhibitory concentrations ($\mu\text{g/ml}$) of marbofloxacin and marbofloxacin–gentamicin (1:1) combination in sixteen *P. aeruginosa* strains on Days 1–8. The original MICs of the strains are shown on Day 1.

		Days							
		1	2	3	4	5	6	7	8
M	MIC _{range}	0.5–16	2–16	2–32	2–64	4–64	4–64	4–128	4–128
	MIC ₅₀	2	4	8	8	8	16	32	64
	MIC ₉₀	8	16	32	32	32	64	129	128
	MIC _{range}	0.5	0.5–4	1–8	1–8	1–8	1–8	1–16	1–32
M:G	MIC ₅₀	1	2	2	2	2	2	4	4
	MIC ₉₀	4	4	8	4	4	8	16	16

M – Marbofloxacin solution

M:G – Marbofloxacin and gentamicin (1:1) solution

Serial passage in subinhibitory concentrations showed a significant increase in the MICs of marbofloxacin after one day. Serial passage in subinhibitory concentrations showed an increase in the MICs of marbofloxacin–gentamicin (1:1) for all strains of *P. aeruginosa* but the MIC increase was much lower than that found for the treatment with marbofloxacin alone. No strain reached an MIC higher than 32 $\mu\text{g/ml}$.

The MIC increase of *P. aeruginosa* strains during the 8 day serial passage is shown in Figure 15.

The multiplication intensity and the absolute increase of the MICs were always lower in case of the marbofloxacin–gentamicin (1:1) combination. In addition, it has to be stated that the original (day 1) results of the combination were much lower, and thus the multiplication factor indicates a much lower absolute increase. Considering these results, it can be established that the marbofloxacin–gentamicin (1:1) combination can significantly hinder the development of resistance in *P. aeruginosa* strains *in vitro*.

The nonlinear trends of MIC values shown in Table 8 were statistically modelled with power functions. The estimated base and exponent parameters are summarised in Table 8.

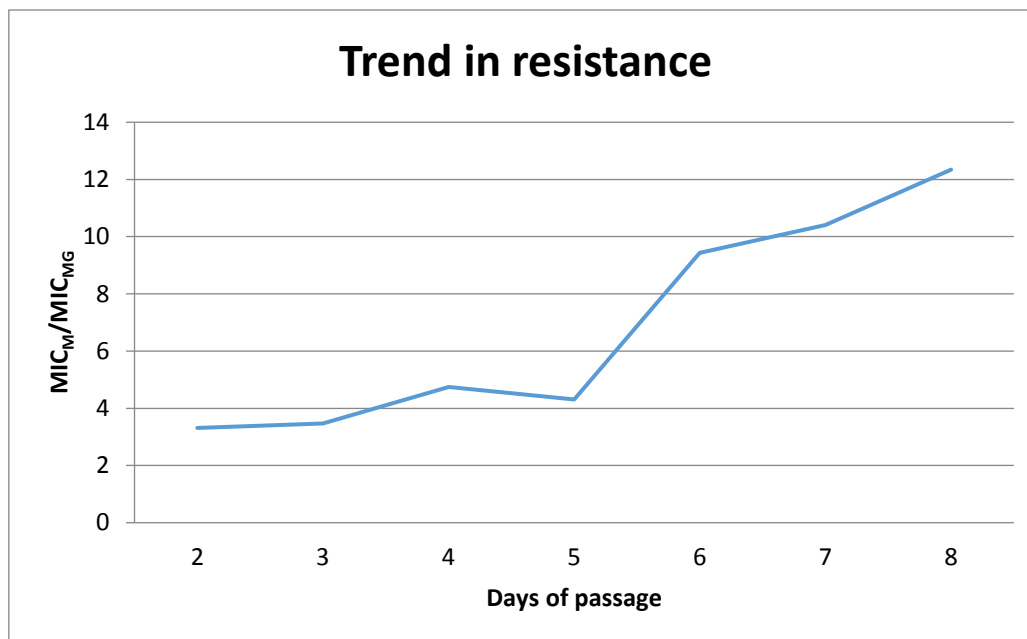


Figure 15. The ratio of average MIC values of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination for *P. aeruginosa* strains during 8 day serial passage period. The graph shows that MIC of marbofloxacin increased significantly as compared to the MICs of the combination

At 5% type I error level base coefficients differed significantly ($p = 0.0009$) between marbofloxacin and marbofloxacin–gentamicin treatments. Exponents were also significantly different at $p < 0.0001$. Consequently, the MIC trend for marbofloxacin - during the trial. Moreover, the ratio of the two trends (M/MG) increased (its Base was higher than 1 and its Exponent was positive).

Table 8. Base and Exponent parameters in the case of marbofloxacin and marbofloxacin + gentamicin treatments

Treatment	Base	Exponent	Trend function
Marbofloxacin	1.74	1.37	$MIC = 1.74 \cdot Day^{1.37}$
Marbofloxacin + Gentamicin	1.04	0.70	$MIC = 1.04 \cdot Day^{0.70}$
*M/MG	1.67	0.67	$MIC = 1.67 \cdot Day^{0.67}$

*M/MG – Quotient of MIC_{marbofloxacin} and MIC_{marbofloxacin and gentamicin}

Significant increase in MIC to marbofloxacin was noted in strain No. 16 that was subjected to PCR and CCCP analysis. The amino acid alterations found in GyrA, GyrB and NfxB of this *in vitro* selected antibiotic resistant strain are listed in Table 9. The marbofloxacin passaged mutant (M16) harboured amino acid change in GyrB in position 466, which importance in fluoroquinolone resistance have been described by Akasaka *et al.* (2001). This strain also contains alterations in NfxB (in 115 and 190 amino acid positions), a regulator protein of the MexCD-OprJ efflux system. The marbofloxacin–gentamicin passaged strain (MG16) harboured change only at position 119 of GyrA. No amino acid changes were detected in ParC, ParE, MexR, MexT and MexZ, respectively.

Analysing the mutations together with the CCCP assay it can be hypothesised that the amino acid changes found in NfxB and GyrB are responsible primarily for MIC increase in the M16 mutant. GyrA mutation in MG16 only caused a minor increase in MICs compared to the M16 strain. The control strain (C16) did not show any decrease in MIC to any antibiotics co-treated with CCCP hypothesising very low efflux pump activity.

Table 9. Effects of CCCP on marbofloxacin and gentamicin MICs on the last day of the study and amino acid changes of *P. aeruginosa* strain No. 16

Strains	MICs (µg/ml)				Mutations			
	Marbofloxacin		Gentamicin		GyrA	GyrB	NfxB	
	-CCCP	+CCCP	-CCCP	+CCCP	119 (119) ^a	466 (464) ^a	115	190
C16	0.5	0.5	0.5	0.5	Ala	Ser	Arg	Ser
M16	32	4	16	8	Ala	Phe	Trp	Ala
MG16	4	4	8	4	Thr	Ser	Arg	Ser

^a The numbers in parentheses are the numbers of the corresponding codons in *E. coli*
 C16 refers to the strain passaged for 8 days without antibiotics
 M16 refers to the strain passaged for 8 days in marbofloxacin
 MG16 refers to the strain passaged for 8 days in marbofloxacin–gentamicin (1:1) combination

Staphylococcus pseudintermedius

The minimum inhibitory concentrations (MICs) of marbofloxacin and the marbofloxacin-gentamicin (1:1) combination in sixteen *S. pseudintermedius* strains during the 7 passages are shown in Table 10.

Table 10. Minimum inhibitory concentrations ($\mu\text{g/ml}$) of marbofloxacin and marbofloxacin–gentamicin (1:1) combination in sixteen *S. pseudintermedius* strains on Days 1–8. The original MICs of the strains are shown on Day 1

		Days							
		1	2	3	4	5	6	7	8
	MIC _{range}	0.03–2	0.03–8	0.03–8	0.125–8	0.5–8	0.5–8	0.5–8	0.5–8
M	MIC ₅₀	0.25	0.5	0.5	1	1	2	2	2
	MIC ₉₀	1	1	1	2	2	4	4	4
	MIC _{range}	0.03–4	0.03–4	0.03–4	0.015–4	0.06–4	0.015–8	0.06–8	0.015–8
M:G	MIC ₅₀	0.125	0.25	0.125	0.125	0.125	0.25	0.25	0.25
	MIC ₉₀	0.5	0.5	0.5	0.5	1	1	1	2

M – Marbofloxacin alone

M:G – Marbofloxacin and gentamicin (1:1) combination

The graphical illustration of the increase in MIC values in case of selected *S. pseudintermedius* strains are shown in Figure 16. The graphs show the multiplication factors of the MIC compared to the original (first day) values.

The nonlinear trends of MIC values shown in Figure 16 were statistically modelled with power functions. The estimated base and exponent parameters are summarised in Table 11.

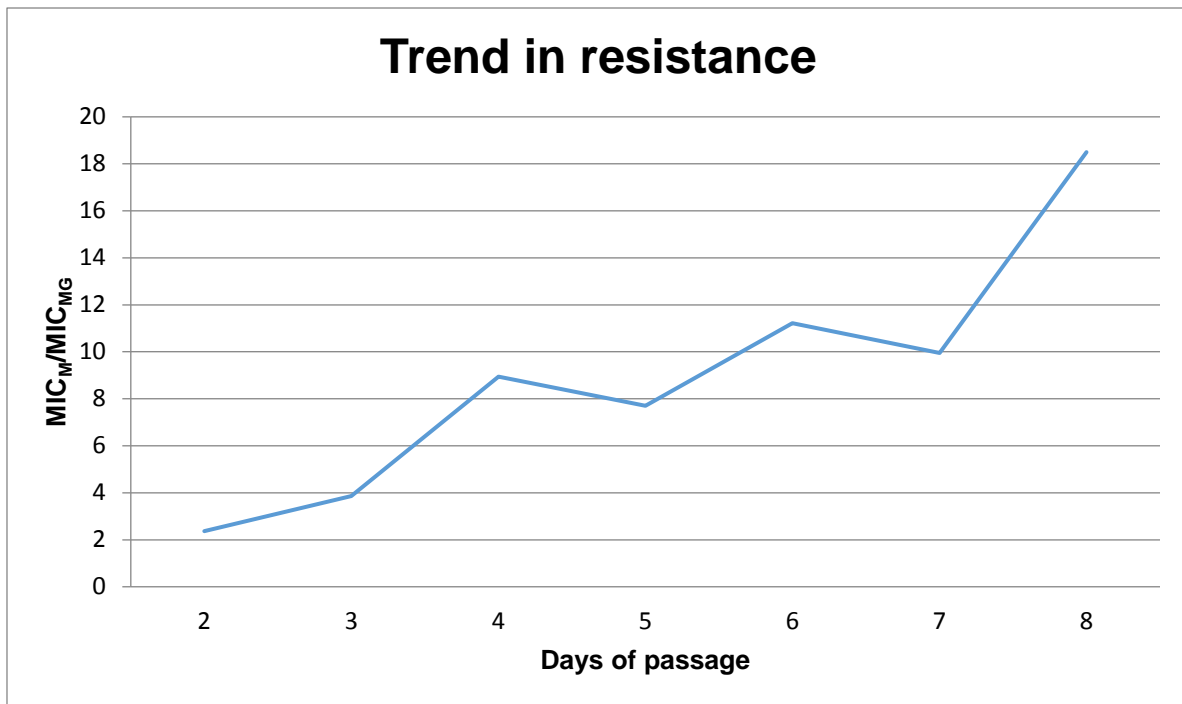


Figure 16. The ratio of average MIC values of marbofloxacin and the marbofloxacin–gentamicin (1:1) combination for *S. pseudintermedius* strains during 8 day serial passage period. The graph shows that MIC of marbofloxacin increased significantly as compared to the MICs of the combination.

Base values did not differ significantly at 5% type I error level between treatments marbofloxacin and marbofloxacin+gentamicin $p=0.4504$. The Exponent of treatment marbofloxacin was significantly positive, $p<0.0001$. The Exponent of marbofloxacin+gentamicin was also significantly positive, $p=0.0005$. The Exponents of the two treatments differed significantly, $p<0.0001$.

Table 11. Base and Exponent parameters in the case of marbofloxacin and marbofloxacin + gentamicin treatments.

Treatment	Base	Exponent	Trend function
Marbofloxacin	0.20	1.10	$MIC = 0.20 \cdot Day^{1.10}$
Marbofloxacin + gentamicin	0.17	0.23	$MIC = 0.17 \cdot Day^{0.23}$
*M/MG	1.15	0.67	$MIC = 1.15 \cdot Day^{0.67}$

*M/MG – Quotient of $MIC_{\text{marbofloxacin}}$ and $MIC_{\text{marbofloxacin and gentamicin}}$

We applied a simultaneous statistical test to determine the days when MIC trend values of marbofloxacin and marbofloxacin+gentamicin differed. Having fixed the family-wise error rate at 5 per cent we obtained no significant difference on the 1st day ($p=0.6570$). However, the trend values of marbofloxacin were significantly greater than that of marbofloxacin+gentamicin on all subsequent days ($p<0.0001$). Family-wise error rate or simultaneous significance level means the error probability is considered simultaneously for all days involved in the trial.

6.4. Susceptibility of *Staphylococcus* spp., *Streptococcus canis* and *Pseudomonas aeruginosa* strains to marbofloxacin and susceptibility determination of *Malassezia pachydermatis* strains to ketoconazole

MIC values in 2010 and 2017–2018

The MIC-values of marbofloxacin were determined for each bacterial strain. CLSI breakpoints were used to determine the ratio of susceptible, moderately susceptible and resistant strains. In the case of marbofloxacin MIC values equal or below 1 µg/ml were considered as susceptible. MIC values of 2 µg/ml were defined as moderately susceptible and values of 4 µg/ml or higher were considered as resistant. The same interpretive standards were used for each bacterial species according to the CLSI recommendations.

The MIC-values of ketoconazole were determined for each fungal strain. CLSI breakpoints were used to determine the ratio of susceptible, moderately susceptible and resistant strains. Isolates with MICs ≤ 0.125 µg/ml were considered susceptible, MICs of 0.25 were considered moderately susceptible and isolates with MICs ≥ 0.5 µg/ml were defined as resistant (CLSI M27-A3. 2008)

The susceptibility of the bacteria and *M. pachydermatis* investigated are shown in Table 12–19.

Table 12. Frequency distribution of *Staphylococcus* spp. in 2010 (n=135)

<i>S. pseudintermedius</i> and <i>S. aureus</i>										
Minimum inhibitory concentration – marbofloxacin (µg/ml)										
>32	32	16	8	4	2	1	0.5	0.25	0.125	0.063
0	0	0	0	1	0	1	4	61	60	8

Table 13. Frequency distribution of *Staphylococcus* spp. in 2017–2018 (n=89)

<i>S. pseudintermedius</i> and <i>S. aureus</i>										
Minimum inhibitory concentration – marbofloxacin (µg/ml)										
>32	32	16	8	4	2	1	0.5	0.25	0.125	≤0.063
0	3	1	0	1	1	4	7	16	3	53

Staphylococcus spp. MIC₉₀ was 0.25 µg/ml of isolates in 2010.

Staphylococcus spp. MIC₉₀ was 1 µg/ml of isolates in 2017–2018.

Table 14. Frequency distribution of *P. aeruginosa* in 2010 (n=56)

<i>Pseudomonas aeruginosa</i>										
Minimum inhibitory concentration – marbofloxacin (µg/ml)										
64	32	16	8	4	2	1	0.5	0.25	0.125	0.063
1	0	3	6	1	17	18	9	1	0	0

Table 15. Frequency distribution of *P. aeruginosa* in 2017–2018 (n=55)

<i>Pseudomonas aeruginosa</i>										
Minimum inhibitory concentration – marbofloxacin (µg/ml)										
64	32	16	8	4	2	1	0.5	0.25	0.125	64
1	1	0	0	9	9	18	4	3	10	0

P. aeruginosa MIC₉₀ was 8 µg/ml of isolates in 2010.

P. aeruginosa MIC₉₀ was 4 µg/ml of isolates in 2017–2018.

Table 16. Frequency distribution of *S. canis* in 2010 (n=43)

<i>Streptococcus canis</i>										
Minimum inhibitory concentration – marbofloxacin (µg/ml)										
64	32	16	8	4	2	1	0.5	0.25	0.125	0.063
0	0	0	0	3	25	13	2	0	0	0

Table 17. Frequency distribution of *S. canis* in 2017–2018 (n=22)

<i>Streptococcus canis</i>										
Minimum inhibitory concentration - marbofloxacin (µg/ml)										
>64	64	32	16	8	4	2	1	0.5	0.25	≤0.125
0	0	0	0	1	1	16	2	1	0	1

Streptococcus canis MIC₉₀ was 2 µg/ml of isolates in 2010.

Streptococcus canis MIC₉₀ was 2 µg/ml of isolates in 2017–2018.

Table 18. Frequency distribution of *M. pachydermatis* in 2010 (n=54)

<i>M. pachydermatis</i>									
Minimum inhibitory concentration - ketoconazole (µg/ml)									
16	8	4	2	1	0.5	0.25	0.125	0.063	0.031
0	0	0	0	0	0	0	2	18	34

Table 19. Frequency distribution of *M. pachydermatis* in 2017–2018 (n=80)

<i>M. pachydermatis</i>									
Minimum inhibitory concentration - ketoconazole (µg/ml)									
16	8	4	2	1	0.5	0.25	0.125	0.063	0.031
0	0	0	0	0	0	1	3	12	64

M. pachydermatis MIC₉₀ was 0.063 µg/ml of isolates in 2010.

M. pachydermatis MIC₉₀ was 0.063 µg/ml of isolates in 2017–2018.

Remarks:

white background: Resistant

pale gray background: Moderately susceptible

dark gray background: Susceptible

6.5. Activity of ketoconazole against biofilm producing and planktonic *Malassezia pachydermatis* strains

All of the tested *M. pachydermatis* isolates were able to produce biofilms on the catheter surface, as confirmed by crystal violet staining and SEM. A SEM picture of an 8 hours old *M. pachydermatis* biofilm is shown in Figure 17.

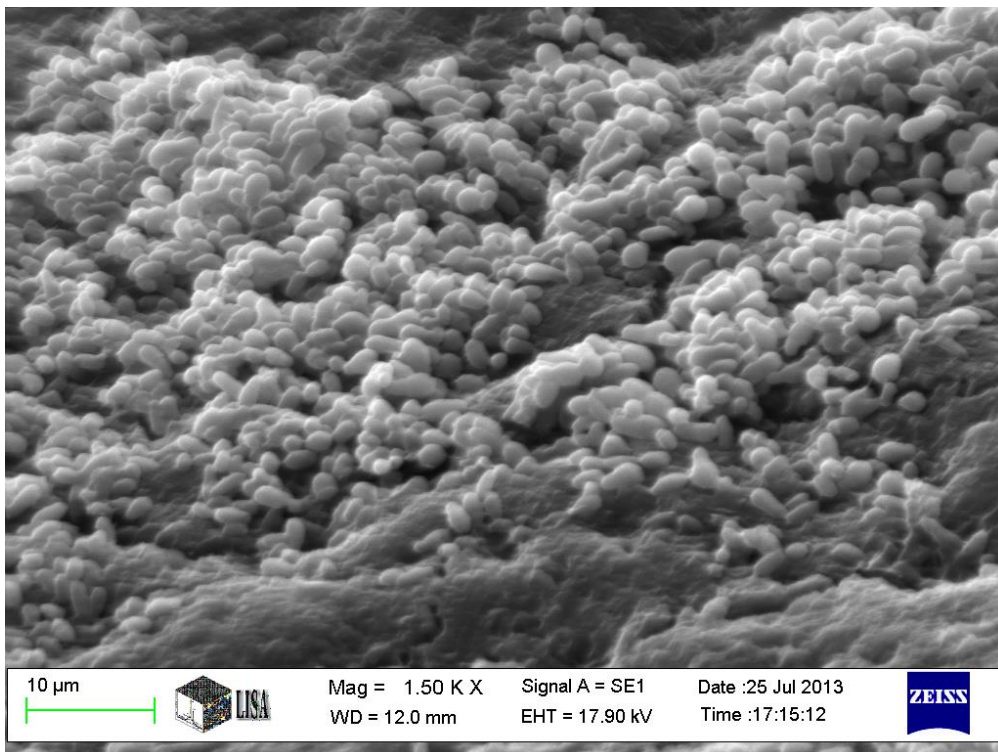


Figure 17. Scanning electron micrograph of an 8 hours old *Malassezia pachydermatis* biofilm (Zeiss Evo MA10; magnification: · 2500)

Determination of MIC_{ketoconazole}-values with the unaided eye in planktonic and biofilm producing systems

The MIC-values of ketoconazole were determined for each yeast strain. The results of fungal growth of the *M. pachydermatis* strains involved in the study are shown in Table 20. In planktonic forms of *M. pachydermatis* strains MIC values ranged from <0.008 to 0.04 µg/ml indicating extreme susceptibility of the yeast to ketoconazole. Most of the strains were even inhibited by the lowest ketoconazole (0.008 µg/ml) concentration.

Table 20. Growth intensities of the biofilm producing forms and planktonic forms of *M. pachydermatis* strains (n=8) at different concentrations of ketoconazole. Left column shows strain number, first line shows ketoconazole concentrations in µg/ml. Odd line numbers (1, 3) include biofilm producing forms while even line numbers (2, 4) include planktonic forms of certain strains.

Strain No./Ketoconazole concentration	25	5	1	0.2	0.04	0.008
Strain No. 3 in biofilm	-	-	+	++	+++	+++
Strain No. 3 without biofilm	-	-	-	-	-	-
Strain No. 9 in biofilm	-	-	++	++	++	+++
Strain No. 9 without biofilm	-	-	-	-	-	-
Strain No. 10 in biofilm	-	-	-	+	++	+++
Strain No. 10 without biofilm	-	-	-	-	-	-
Strain No. 13 in biofilm	-	-	-	-	+++	+++
Strain No. 13 without biofilm	-	-	-	-	-	-
Strain No. 16 in biofilm	-	-	++	++	+++	+++
Strain No. 16 without biofilm	-	-	-	-	-	-
Strain No. 18 in biofilm	-	-	++	+++	+++	+++
Strain No. 18 without biofilm	-	-	-	-	-	+
Strain No. 20 in biofilm	-	+	++	++	+++	+++
Strain No. 20 without biofilm	-	-	-	-	-	-
Strain No. 43 in biofilm	-	-	+	++	++	+++
Strain No. 43 without biofilm	-	-	-	-	-	-

+++ pronounced growth of the yeast, ++ = moderate growth of the yeast, + low growth potency of the yeast, = no growth

In biofilm producing forms of *M. pachydermatis* the MIC values were significantly higher, ranging from 0.2 to 25 µg/ml. Our data indicate a 25–3125 times increase in the MIC values in the biofilm producing systems compared to the planktonic yeasts when determined by the unaided eye.

Determination of MIC_{ketoconazole}-values with the Celltiter96® Aqueous Proliferation Assay (MTS) in planktonic and biofilm producing systems

The absorbance results obtained by ELISA reader at 490 nm are shown in Table 21. These are corrected values, taking into account the absorbance blank samples. The absorbance values are in positive correlation with the growth and viability of *M. pachydermatis* strains. All measurements were repeated three times, Table 20. contains the mean values. Light grey blocks represent weak-moderate, dark grey represents pronounced growth. Threshold absorbance values were 0.08 and 0.20 for weak-moderate and pronounced growth, respectively.

In planktonic forms of *M. pachydermatis* strains MIC values ranged from <0.008 to 0.04 µg/ml to ketoconazole, in consonance with our previous results (unaided eye determination). Most of the strains were even inhibited by the lowest ketoconazole (0.008 µg/ml) concentration. These wells usually did not contain any viable organisms, thus not only the MIC, but the MFC (minimum fungicidal concentration) could be determined. According out result the MFC values in these strains were also between <0.008 and 0.04 µg/ml.

In biofilm producing forms of *M. pachydermatis* the MIC values were significantly higher, ranging from 0.2 to >25 µg/ml. In strain 20 growth could not be inhibited by the 25 µg/ml concentration, thus MIC and MFC were above this value.

Our data indicate a similar 25–3125 times increase in the MIC values in the biofilm producing systems compared to the planktonic yeasts when determined by the *Celltiter*® Assay. In strain 20 however, increase in MIC could even be higher, but not determined in this study.

Table 21. Absorption values of the biofilm producing forms and planktonic forms of *M. pachydermatis* strains (n=8) at different concentrations of ketoconazole. Left column shows strain number, first line shows ketoconazole concentrations in µg/ml. Odd line numbers (1, 3) include biofilm producing forms while even line numbers (2, 4) include planktonic forms of certain strains

Strain No./Ketoconazole concentration	25	5	1	0.2	0.04	0.008
Strain No. 3 in biofilm	0.059	0.036	0.042	0.094	0.322	1.020
Strain No. 3 without biofilm	0.075	0.048	0.028	0.035	0.054	0.098
Strain No. 9 in biofilm	0.099	0.098	0.097	0.132	0.271	1.231
Strain No. 9 without biofilm	0.076	0.047	0.035	0.040	0.049	0.058
Strain No. 10 in biofilm	0.005	0.006	0.077	0.031	0.247	1.258
Strain No. 10 without biofilm	0.058	0.049	0.040	0.035	0.047	0.076
Strain No. 13 in biofilm	0.037	0.059	0.050	0.037	1.373	1.913
Strain No. 13 without biofilm	0.047	0.057	0.062	0.041	0.061	0.021
Strain No. 16 in biofilm	0.007	0.019	0.217	0.203	0.924	1.717
Strain No. 16 without biofilm	0.041	0.051	0.008	0.013	0.022	0.197
Strain No. 18 in biofilm	0.033	0.014	0.210	0.774	1.626	1.780
Strain No. 18 without biofilm	0.030	0.041	0.041	0.022	0.049	0.150
Strain No. 20 in biofilm	0.061	0.085	0.126	0.133	1.037	1.647
Strain No. 20 without biofilm	0.061	0.059	0.030	0.042	0.068	0.096
Strain No. 43 in biofilm	0.023	0.034	0.230	1.051	1.112	1.725
Strain No. 43 without biofilm	0.019	0.002	0.032	0.061	0.012	0.022

7. Conclusions

Multidrug resistant and extensively drug-resistant *P. aeruginosa* strains became abundant in the last decades in Europe and cause frequent therapeutic failure in veterinary and human practice alike (Souli *et al.*, 2008; Rubin *et al.*, 2008). Taking into account the low permeability of the cell wall and the presence of efflux pumps (Li *et al.*, 1995), the broad range of β -lactamases produced, biofilm production (Agarwal *et al.*, 2005), and the high genetic versatility of this species, only a very limited number of antimicrobials are at the clinicians' disposal. Enrofloxacin and marbofloxacin are licensed only for veterinary use, the latter being more active against *P. aeruginosa* (Müller and Horn, 2009; Kroemer *et al.*, 2013). According to a publication 349 *P. aeruginosa* strains isolated from dogs and cats were investigated and 23% of these proved to be resistant to marbofloxacin, a lower ratio compared to our results (36.8%). Regarding gentamicin 65.2% incidence of susceptibility to the aminoglycoside among *Pseudomonas* spp. isolated from chronic otitis externa cases in dogs were reported (Martin Barrasa *et al.*, 2000). In another publication 83.1% of 183 *P. aeruginosa* strains susceptible to gentamicin were reported (Šeol *et al.*, 2002). In our study 89.7% of the isolates were susceptible to gentamicin. There were 3 gentamicin resistant strains that were also resistant to marbofloxacin. Two of these strains proved to be susceptible to the marbofloxacin–gentamicin combination and the MIC of the third strain was also decreased. Thus, those multiresistant strains resistant to the single antimicrobial therapy can be eliminated by the application of marbofloxacin and gentamicin combination.

Resistance in *Pseudomonas aeruginosa* can develop even during therapy with most of the antimicrobials, including fluoroquinolones (Gilbert *et al.*, 2001; Brothers *et al.*, 2002). Multiresistant, extensively drug-resistant and panresistant strains of *P. aeruginosa* have already been reported, and a number of works found that there is no sole antimicrobial that has 100% activity against this bacterium (Timurkaynak *et al.*, 2006; Souli *et al.*, 2008; Tsutsui *et al.*, 2011). According to a publication in 2000 it was reported an incidence of 39–42% multiresistant *P. aeruginosa* isolated from domestic animals between 1989 and 1997 (Normand *et al.*, 2000). In order to kill these multiresistant organisms and delay the development of resistance, a synergistic combination of two bactericidal antibiotics is often required as these might be able to

break up resistance in the microorganism (Fish *et al.*, 2002). The combination of gentamicin and ciprofloxacin improved survival rate in postsurgical septicaemia in humans (Ho *et al.*, 2009). The *in vitro* interactions between enrofloxacin and gentamicin in 7 *P. aeruginosa* strains also was investigated. The fluoroquinolone and the aminoglycoside showed synergy in 5 and additive effect in 2 isolates (Anca *et al.*, 2007).

The results of our study demonstrate synergy between marbofloxacin and gentamicin in 33 (48.5%) isolates, addition and indifference in 31 (45.6%) and 4 (5.9%) isolates, respectively. Analysing the FIC values in all of the isolates, the mean FIC index proved to be 0.546 that describes partial synergy between the substances. In a study the mean FIC value of 0.442 indicated full synergy between enrofloxacin and gentamicin (Anca *et al.*, 2007). The reason of this difference compared to our results may be the lower activity of enrofloxacin against *P. aeruginosa* that could be enhanced significantly by the addition of gentamicin, or just the small number of isolates examined.

The underlying mechanism of synergy between fluoroquinolones and aminoglycosides has not yet been elucidated. Fluoroquinolone resistance is attributed to increased efflux pump activity and/or chromosomal mutations in the *gyrA* or *parC* genes encoding DNA *gyrA*se or topoisomerase IV (Tejedor *et al.*, 2003). Either of these changes can lead to low or high level resistance, and are frequently observed together in resistant strains. As aminoglycosides cause misreading of the genetic code resulting in synthesis of mistranslated proteins, this phenomenon might influence efflux pump activity and expression, leading to decreased survival in the presence of the antimicrobials. Although it might be plasmid encoded (Szmolka *et al.*, 2011), fluoroquinolone resistance is primarily chromosomal, while the genes encoding aminoglycoside modifying enzymes are usually found on plasmids (Shaw *et al.*, 1993). Thus, plasmid and chromosomal resistance mechanisms are usually required jointly in a microorganism to achieve high level resistance to the combination of marbofloxacin and gentamicin.

The usage of fluoroquinolones in veterinary dermatology is recommended in those cases when susceptibility testing indicates that the pathogen is resistant to other classes of antimicrobials (Prescott *et al.*, 2002; Beco *et al.*, 2013). Some authors would recommend systemic usage of these antimicrobials instead of topical administration for treatment of external otitis or pyoderma (Ihrke *et al.*, 1999), however, a much higher

concentration can be achieved with the latter method. As mentioned earlier, selection of resistant organisms is much less likely when fluoroquinolones are used in combination, as several mutation mechanisms should occur at the same time to achieve a high level resistance in *P. aeruginosa* that can lead to therapeutic failure. However, non-prudent use of fluoroquinolones and consequent development of resistance may lead to public health risk as bacteria can be reservoirs of antibiotic-resistant bacteria (Guardabassi *et al.*, 2004; Murphy *et al.*, 2009; Beco *et al.*, 2013).

In conclusion, full synergy has been found between marbofloxacin and gentamicin in 48.5% of the *P. aeruginosa* strains investigated, with a mean FIC index of 0.546 indicating only partial synergy for all of the strains. The threshold of full synergy is at 0.5 FIC, and no antagonistic effect was observed in any of the strains, therefore, according to our results the justified and targeted usage of the marbofloxacin/gentamicin combination in infections caused by *P. aeruginosa* in the veterinary field may yield beneficial results, especially in topical products where the toxic effects of gentamicin might be negated if the tympanic membrane is intact.

Ear drops containing gentamicin are often used in human and veterinary medicine for the treatment of external otitis. Because of a potential irreversible ototoxic effect (Matz *et al.*, 2000), gentamicin-containing products cannot be used in cases with perforated tympanic membrane (Rosenfeld *et al.*, 2006). Although our studies were conducted on monolayer IPEC-J2 cells our results suggest that gentamicin alone or supplemented with 1% DMSO cannot penetrate through the intact tympanic membrane into the inner ear. The tympanic membrane is coated with a striated multilayer epithelium, thus offering further protection from the potential ototoxic effect of gentamicin. The tight barrier due to IPEC-J2 cells grown in a monolayer and linked by tight junction proteins is less complex than the multi-layered tympanic membrane therefore our approach is over-predictive. Furthermore, our data show that the addition of the organic solvent DMSO did not alter the transport of gentamicin through an intestinal epithelial monolayer, hence it might not enhance the oral absorption of gentamicin if ingested accidentally. Whether our *in vitro* results can be extrapolated to the *in vivo* situation remains to be investigated.

In conclusion, gentamicin showed poor paracellular permeation across the IPEC-J2 monolayer. Addition of 1% DMSO did not disrupt paracellular integrity and it did alter the transport of gentamicin. Our results indicate that inclusion of DMSO in this low

concentration as part of drug formulation does not promote oral absorption of gentamicin and it does not enhance the incidence of ototoxic effects caused by gentamicin in ear drop application. In the future, gentamicin can be used for detecting epithelial barrier dysfunction during LPS-induced bacterial infection or acute oxidative stress when chromatographic separation and quantitative fluorometric determination of gentamicin collected from basolateral compartment of IPEC-J2 cell monolayer is performed.

Results of the present study revealed that the ratio of resistant *P. aeruginosa* strains was significantly lower when passaged for 8 days in sublethal concentrations of gentamicin and marbofloxacin solution as compared to sublethal concentrations of marbofloxacin solution alone. Although the professional guidelines clearly oppose the use of antibiotics in concentrations lower than the therapeutic dose and/or for durations shorter than the recommended period, this misuse cannot be considered negligible (Hughes *et al.*, 2012; Isturiz, 2010) and probably cannot be avoided. The present study contributes to understanding the extent to which fixed combinations used in the wrong way and/or for the inappropriate period of time contribute to the development of resistant strains. Further studies should be done on other fixed combinations and other potentially pathogenic bacteria. It would also be advisable to test the effect of serial passage on the resistance profile of the tested bacteria against antibiotics that were not applied in the serial passage.

Apart from stating that bacterial resistance increases (Latife, 2008; Mohr and Jones, 2004), the search for new antibacterial active ingredients follows this problem only to a very limited extent. Several studies drew attention to the escalation of resistance and pointed out the use of fixed combinations as a possible alternative to control this increasing resistance (Milatovic and Braveny, 1987; Mouton, 1999). Other studies demonstrated that combination treatments do not provide a significant advantage over a carefully chosen monotherapy in average cases or in cases where the bacterium shows proper sensitivity to the appropriate active ingredient used in monotherapy (Bowers *et al.*, 2013; Leibovici *et al.*, 1997). However, in severe cases, particularly when the infection is caused by a Gram-negative bacterium, the superiority of combination therapies has been reported, especially when the therapy was started early (Traugott *et al.*, 2011). A properly chosen combination treatment is still almost the only option against microbial resistance (Lim *et al.*, 2011; Poulikakos *et al.*, 2014).

Another advantage of combination therapy is that the resistance of Gram-negative bacteria will develop more slowly (Traugott *et al.*, 2011), which is confirmed by results from the present study. It is also important to draw attention to the need of exploring the potential possibilities of interactions between active ingredients (Jerzsele and Pászti-Gere, 2015; Mărculescu *et al.*, 2007; Traugott *et al.*, 2011), and to determine the interactions that could be used effectively in the antibacterial strategy. The study of these interactions requires further investigations.

Average multiplication of the MICs on day 8 passaged in marbofloxacin alone and the marbofloxacin–gentamicin (1:1) combination were 11.67 compared to 3.00, respectively. In addition it has to be stated that the original (day 1) results of the combination were usually lower, thus the multiplication factor indicates a much lower absolute increase. All of our results showed highly significant differences ($p < 0.0001$). Considering these results, it can be pronounced that the marbofloxacin–gentamicin (1:1) combination can significantly hinder the development of fluoroquinolone resistance in *S. pseudintermedius* bacteria.

Table 22 shows MIC values of different *Staphylococcus* spp., *Streptococcus canis*, *P. aeruginosa* and *Malassezia pachydermatis* strains isolated from dog skin concerned by dermatitis in 2010 in comparison with MICs gained from the same sources in 2017–2018. The ratio of sensitive microbial population is given in percent. As can be seen *Staphylococcus* spp., *S. canis*, *P. aeruginosa* and *M. pachydermatis* show definite susceptibility to marbofloxacin and ketoconazole.

Besides that on significant difference were observed in MICs between 2010 and 2017–2018. Based on these results it can be stated, that marbofloxacin and ketoconazole are still been valuable and useful antibiotic against the most important bacteria and *M. pachydermatis*.

Table 22. Summary about the bacteria, fungi, their number of isolates, the ratio of susceptible strains to marbofloxacin, ketoconazole

	2010		2017–2018	
	No of Isolates	Sensitive to marbofloxacin	No of Isolates	Sensitive to marbofloxacin
<i>Staphylococcus spp.</i>	135	99.30%	89	94.38%
<i>S. canis</i>	43	93.02%	22	90.90%
<i>P. aeruginosa</i>	56	80.35%	55	80.00%
		Sensitive to ketoconazole		Sensitive to ketoconazole
<i>M. pachydermatis</i>	54	100%	80	98.75%

According to our results it can be stated, that *M. pachydermatis* strains showed very high susceptibility to ketoconazole in their planktonic (suspended) forms. However, when planted on surface catheters to produce biofilms their susceptibility was changed significantly. Our data achieved with the unaided eye determination and the *Celltiter96® Aqueous Proliferation Assay* measurements were mainly in consonance. These data highlighted that biofilm producing forms of a yeast strain can show 25–3125 times decrease in susceptibility (25–3125 times increase in MIC) compared to the planktonic forms of the respective strain.

These data can be of utmost importance when evaluating the *in vivo* efficacy of an antifungal. *In vitro* susceptibility data can be essentially changed in living systems. Thus, the effect of ketoconazole can be much less pronounced against these strains and forms of yeasts. According to these results, a much higher concentration of ketoconazole is recommended in dermatological products than the average MIC₉₀ values, if *M. pachydermatis* infection is suspected. This can inhibit and even kill biofilm producing strains, thereby decreasing the development of resistance in these fungi.

8. New scientific results

Synergistic interaction between marbofloxacin and gentamicin against *Pseudomonas aeruginosa* were discovered in canine otitis externa isolates.

It was proven that addition of 1% DMSO to gentamicin solutions did not disrupt paracellular integrity of IPEC-J2 monolayer and it did not alter the transport of gentamicin.

Examination revealed that the ratio of resistant *Pseudomonas aeruginosa* strains was significantly lower when passaged for 8 days in sublethal concentrations of the combination of gentamicin and marbofloxacin when compared to sublethal concentrations of marbofloxacin alone.

Our study revealed that the ratio of resistant *Staphylococcus pseudintermedius* strains was significantly lower when passaged for 8 days in sublethal concentrations of the combination of gentamicin and marbofloxacin when compared to sublethal concentrations of marbofloxacin alone.

In our examination we did not find significant differences in MIC values of staphylococci, *Streptococcus canis*, *Pseudomonas aeruginosa* against marbofloxacin and *Malassezia pachydermatis* against ketoconazole between 2010 and 2017–2018.

According to our results it can be stated, that *Malassezia pachydermatis* strains collected from Hungarian dogs suffering from clinical signs of malassezia infections of the skin or the ear showed 25–3125 times decrease in susceptibility when presented in biofilms (25–3125 times increase in MIC) compared to the planktonic forms of the respective strain.

9. References

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10. Relating author's publications

10.1. Publications related to the topic of the present dissertation

10.1.1. Full Text papers in peer-reviewed journal

Gyetvai B., Jerzsele Á., Pászti-Gere E., Nagy G., Gálfi P.: Gentamicin sulphate permeation through porcine intestinal epithelial cell monolayer. *Acta Veterinaria Hungarica* 63 (1), pp. 60–68 2015.

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10.1.2. Oral presentations on Hungarian national conferences

Gyetvai B., Csere I., Szaniszló F., Lang Zs., Gálfi P., Csikó Gy.: Marbofloxacin és ketokonazol hatóanyag tartalmú készítmények hatékonyságának vizsgálata pozitív kontrollkészítménnyel szemben. *Akadémiai Beszámolók* 2013.

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10.2. Publications not related to the topic of the present dissertation

10.2.1. Full Text papers in peer-reviewed journal

Gyetvai B., Lang Zs., Csere I., Kun Cs.: Embrionális oligopeptidkeverék (Humanofort®) per os hatásának vizsgálata kutyák krónikus fájdalommal kísért, gyulladásos mozgásszervi zavarainak konzervatív kezelésében. Magyar Állatorvosok Lapja 135:(12) 746-750. 2013.

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